Published monthly for the Scandinavian Physiological Society

ACTA PHYSIOLOGICA SCANDINAVICA

VOLUME 94 NUMBER 1 May 1975

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Acta Physiologica Scandinavica
Karolinska Institutet
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rinted in Sweden by Almqvist & Wiksell Uppsala 1975

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second millisecond	s ms
mole millimole micro-mole nanomole picomole	mol mmol μmol nmol pmol
meter millimeter micrometer nanometer	m mm μm nm
candela	cd
steradian	sr
hertz (frequency)	Hz (s^{-1})
newton (force)	N (kg m/s^2)
pascal (pressure)	Pa (N/m^2)
joule (energy)	J (N m)
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(kilopond)	kp (9 81 N)
(millimeters of mercury)	mm Hg (1 333 bar)
(millibar)	mbar (100 Pa)
curie	Ci
liter milliliter microliter	l ml μl
degree Celsius	°C

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Almqvist & Wiksell Tryckeri AB
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Acta physiol scand 1975 94 1-7
Department of Physiology and Department of Obstetrics and Gynecology
University of Goteborg Sweden

A Comparison between Direct and Indirect Measurements of Blood Flow in the Follicular Ovary of the Rabbit

By

PER OLOF JANSON and GUNNAR SELSTAM

Received 1 July 1974

Abstract

JANSON P O and G SELSTAM *A comparison between direct and indirect measurements of blood flow in the follicular ovary of the rabbit* Acta physiol scand 1975 94 1-7

A comparison was carried out between d rect and indirect measurements of blood flow in follicular ovaries of anaesthetized laparotomized rabbits Direct measurements by timed quant tative sampling of ovarian venous blood yielded blood flow values 3 times higher than those found with the use of 15 / m radioactive microspheres However when simulating the surgical trauma associated with direct measurements the microsphere technique gave the same high ovar an flow values indicat ng that this type of trauma caused ovarian hyperemia It is concluded that direct measurements of blood flow as performed in the present study are unsuitable for invest gations of circulatory events in the ovary Mechanically induced ovarian vasodilatation is p obably a factor which helps to explain the diverging data on blood flow obtained with d rect and indirect methods The rad oactive microsphere techn que appears to provide a means for reasonably reliable ovarian blood flow measurements in laboratory an mals

Conflicting data have been presented in the literature concerning the magnitude of ovarian blood flow In the sheep for instance direct measurements after cannulation of the ovarian vein have yielded blood flow values about ten times higher than some indirect techniques such as krypton clearance and indicator dilution (Setchell 1969 Mattner and Thorburn 1969)

The present investigation was undertaken to analyze some of the possible reasons for the diverging results in earlier flow measurements Direct measurements of venous outflow were compared with indirect measurements using radioactive microspheres A study was performed under standardized conditions in anaesthetized sexually mature virgin rabbits The aim of the study was to find a method which permits correct measurements of blood flow in this type of ovaries under physiological conditions

Materials and Methods

An mals

Female albino Swed h Land rabb ts 5-6 months old and weighing ~3 kg, were used They had not previously been mated and thei ovaries contained foll cles and had a mean we ght of 91±4 mg The animals we e deprived of food 12- 4 h before the exper ment

TABLE I. Direct measurements of blood flow of the left ovary in [illegible] rabbits[a] Values are expressed as Mean ± S.E.

n	Ovarian weight (mg)	Mean arterial blood pressure (mm Hg)	Blood flow in left ovarian vein[b] (ml/min)		Ovarian blood flow (ml/100 g/min)
			Before clamping	After clamping	
[illegible]	54 ± 12	95 ± 4	0.71 ± 0.07	0.29 ± 0.4	44 ± 07

[a] Connections between ovarian and uterine vessels ligated on the left side

[b] Measurements performed immediately before and after clamping of ovarian vessels at the hilus. Difference in flow rate considered to reflect left ovarian blood flow

Group no. 1 had the vascular supply to both ovaries intact and the area round the ovaries was not manipulated.

Group no. 2 had the ovario-uterine vascular connections ligated and divided on the left side 10 minutes prior to the measurement. The right ovary was left intact.

Group no. 3 had bilaterally intact vascular supply but the left ovary was gently manipulated for 10 s by squeezing it between thumb and index finger 10 min before the measurements.

Table II summarizes the data from these measurements. There were no statistically significant differences in blood flow rate per unit of weight between the two ovaries of the first group. Neither did the flow rate of the right, untouched ovary of the other two groups differ significantly from those of the first group. Ligation of the ovario-uterine vessels or just manipulating the ovary without ligatures of the vessels caused a threefold increase in [illegible] blood flow as measured by this technique 10 min after the mechanical procedure. The mean blood flow to the untouched ovaries of the 15 rabbits was 155 ± 12 ml/100 g/min (21 ovaries).

The renal blood flow measured in the caudal parts of both kidneys in each rabbit was

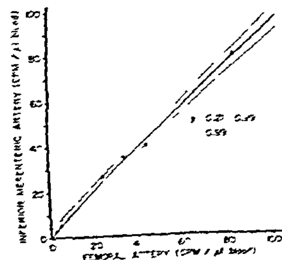


FIG. [illegible] Comparison between amounts of Yttrium-[illegible] labelled 15 µm microspheres (expressed as radioactivity counts in [illegible]) withdrawn simultaneously from the [illegible] in left ventricular injection of [illegible] The [illegible] for differences [illegible] drawn. Regression line was calculated and [illegible] confidence limits [illegible] indicated [illegible] correlation coefficient.

TABLE II Blood flow to follicular ovaries and kidneys as measured by *15±5 µm* [1] Ytterbium labelled microspheres in anaesthetized laparotomized rabbits All values are expressed as Mean ±S E

Group no	n	Experimental model	Mean arterial blood pressure (mm Hg)	Ovarian weight (mg)		Ovarian blood flow (ml 100 g × min)		Renal blood flow (ml/100 g × min)	
				Right	Left	Right	Left	Right	Left
1	6	Intact connections between ovarian and uterine vessels on both sides	86±6	9?±7	96±9	160±23	173±20	287±38	279±36
2	5	Connections between left ovarian and uterine vessels ligated Connections on right side intact	78±8	75±8	74±8	144±37	460±61[a]	303±46	305±44
3	4	Intact connections between ovarian and uterine vessels on both sides Left ovary manipulated[b]	89±8	114±11	108±8	136±	436±96[a]	Not measured	Not measured

[a] $p<0.05$ as compared to the right side
[b] Squeezed between thumb and index finger for 10 s 10 min before measurement

295±20 ml/100 g × min (22 kidneys in 11 rabbits) with no significant differences in flow rate between the left and the right side Ligation of vessels around the left ovary inevitably involves manipulation also of the adjacent left kidney but as indicated by the data from Group no 2 (Table II) this procedure did not measurably influence renal blood flow

Discussion

Direct measurements of blood flow from an intraabdominal organ with a complex vascular supply inevitably involves laparotomy and mechanical interference with the organ and its vessels This may result in unphysiological blood flow values Goding *et al* (1972) reviewing results from various studies of ovarian blood flow in the sheep by direct venous cannulation suggested that one important source of error in such measurements might be a reduction in ovarian blood flow caused either by an increase in venous pressure or a mechanically induced spasm of the ovarian artery This view finds support in a recent study carried out in this laboratory of blood flow in the heavily luteinized ovaries of the pseudopregnant rabbit (Janson and Albrecht 1975) In that study direct measurements by venous cannulation resulted in almost 3 times lower values than indirect measurements using radioactive microspheres In the present study of the *follicular* ovary the vein was transected eliminating the risk of an increased venous pressure When this technique was compared to indirect measurements in intact ovaries using radioactive microspheres the former technique resulted in 3 times higher values than the latter To analyze whether the discrepancy in flow values was

produced by the procedures involved in direct blood sampling, *i.e.* ligation of ovario uterine blood vessels a preparation for direct blood flow measurement was simulated and ovarian blood flow was measured in the preparation by the use of microspheres. In this preparation there was good agreement between results from the two techniques the ovarian blood flow values being three times higher than in intact ovaries. If the ovario-uterine vascular connections were left intact while the ovary was bluntly manipulated the ovarian blood flow as measured with microspheres was increased to the same level as in the ovaries whose ovario-uterine connections were ligated. These data indicate that even gentle handling of the ovary causes a considerable increase in blood flow. The sensitivity of the follicular ovary to manipulation is a definite drawback when using indirect techniques for flow measurements involving a local application of indicator directly into the ovarian tissue. Such techniques also have other drawbacks when applied to the ovary which is an organ with several compartments with different perfusion rates (for a discussion see Lassen and Larsen 1972).

The present modification of the microsphere technique for blood flow measurement involves no local trauma to the ovaries and the technique can even be performed in conscious animals (Neutze *et al.* 1968). The present study and similar investigations in this laboratory have shown that the intracardiac infusions of microspheres cause no significant changes in blood pressure cardiac output and heart rate. The spheres have been shown to distribute homogenously in the aortic blood stream as indicated by the results from the present study and when reaching the ovary they do not shunt to the venous side (Ahrén *et al.* 1974). The number of spheres trapped in the ovaries of the present study permits ovarian blood flow measurements with a precision of 5–10% at a 95% confidence level. Ovarian blood flow can thus be measured using microsphere technique with a reasonable accuracy in a small experimental animal like the rabbit and the technique seems to be suitable for further studies of ovarian blood flow regulation.

The mechanism of the increase in ovarian blood flow evoked by the preparation procedure for direct blood flow measurement is unclear. Damage to vessels and vasomotor nerves may cause the release of vasoactive substances such as adenosine compounds potassium ions and biogenic amines (Folkow and Neil 1971). Recently a similar mechanically induced vasodilatation has been demonstrated in the small intestine (Biber *et al.* 1971) probably evoked *via* a local nervous reflex and probably involving 5 hydroxy tryptamine as a mediating substance. This substance was also suggested to be involved in acute intestinal vasodilatation in response to gastrointestinal hormones (Biber *et al.* 1974). In the ovary it has been shown that certain gonadotrophic hormones induce acute and marked hyperemia (Ellis 1961 Wurtman 1964) in which both nervous and humoral factors have been suggested to be involved. An investigation of the mechanisms of ovarian hyperemia in response to different stimuli using a reliable technique for blood flow determination is of interest and might possibly give new insight into the mechanism of action of gonadotrophins on the ovary.

This research was sponsored by grants from the Swedish Medical Research Council (B74-03X 7 10C), the National Institutes of Health USPH (5RO1 HD 0?95-0?) Göteborgs Läkaresällskap Greta and Harald Jeansson s Foundation and the Faculty of Medicine University of Göteborg. We are indebted to Professor Kurt Ahrén for valuable suggestions. Skilful technical assistance was given by Miss Kerstin Gustafson.

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Acta physiol scand 1975 94 8–13
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The Release of Prostaglandin-Like Substances during Platelet Aggregation and Pulmonary Microembolism

By

J VAAGE and PRISCILLA J PIPER

Received 11 November 1974

Abstract

VAAGE, J and P J PIPER *The release of prostaglandin like substances during platelet aggregation and pulmonary microembolism* Acta physiol scand 1975 94 8–13

During pulmonary microembolism subsequent to induced platelet aggregation *in vivo* a pressor response is elicited in the lungs due to the release of vasoactive substances During the perfusion of isolated cat lungs with whole blood platelet aggregation induced by collagen released prostaglandin like substances (PG LS) and a rabbit aorta contracting substance (RCS) as measured by continuous bioassay of the venous effluent This release occurred in parallel to the pulmonary pressor response The same amount of PG LS and RCS was released when the lungs were replaced by a blood filter to trap platelet aggregates Apparently PG LS and RCS are released from platelets during their aggregation As E and F type PGs are rapidly inactivated in the pulmonary circulation there must either be an equal generation of PGs in the lungs themselves, or rather the release of PGs from platelets must occur distal to the inactivation mechanism without any additional PG release from the lungs The exact role of PGs in the lung responses to intravascular platelet aggregation is not clear

Circulating blood platelets may attain increased adhesiveness and aggregability and they may even aggregate during pathophysiological states (Dhall *et al* 1969 Ljungquist 1973) Such platelets and platelet aggregates will to a large extent be trapped in the pulmonary vascular bed (Ljungquist Bergentz and Lewis 1971) Pulmonary retention of platelets and platelet aggregates has again been claimed to cause lung dysfunction and to be a component in the pathogenesis of acute respiratory distress (Bø and Hognestad 1973 Blaisdell and Schlobohm 1973 Blaisdell 1974) When platelet aggregation is induced *in vivo* a powerful constriction of the smooth muscles in both lung vessels and in airways is elicited This constriction is probably caused by the release of vasoactive substances from platelets or from the lungs (Rådegran and McAslan 1972, Vaage Bø and Hognestad 1974) Prostaglandins (PGs) may be important in this connection since PGs (E_2 and F_2) are released in platelet rich plasma during aggregation (Smith and Willis 1970 Smith *et al* 1973) Piper and Vane (1971) have also demonstrated release of PGs from lungs after various mechanical stimuli including embolism with various non biological particles (0 8–120 μm diameter)

In the present work we investigated whether PGs are released in parallel with the pul-

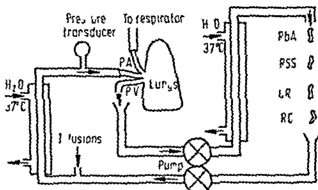


Fig 1 Diagram of the apparatus for perfusion of isolated cat lungs Ventilated cat lungs were perfused with blood at 37 C Perfusion pressure was measured in the pulmonary artery (PA) The venous effluent was reheated (37 C) and pumped to superfuse 4 assay tissues rabbit aorta (RbA) rat stomach strip (RSS) chick rectum (CR) and rat colon (RC) Then the blood was pumped back to the lungs A blood filter was sometimes used in place of lungs in the circuit

monary pressor response elicited in the lungs by pulmonary microembolism with induced platelet aggregates Additionally we wanted to obtain information whether PGs are released from platelets only from lungs only or from both after induced intravascular platelet aggregation Since rabbit aorta contracting substance (RCS) is released together with PGs we also decided to investigate the release of this substance

Methods

The method used was a modification of the blood bathed organ technique (Vane 1964 Piper and Vane 1969) Cats weighing 2.5–4.0 kg were anesthetized with pentobarbitone sodium 30–40 mg/kg given intraperitoneally and then heparinized (500 IU/kg) In each experiment one cat was used as a blood donor and exsanguinated by cardiac puncture Another cat was initially bled 50 ml from a catheter in the carotid artery to obtain additional blood and then served as lung donor A tracheotomy was performed and continuous positive pressure ventilation commenced (5 ml/stroke 22 strokes min) This ventilation is close to the normal one for cats of this size (Vaage unpublished observations) The end expiratory tracheal pressure was kept at about 2 cm H_2O by means of a water seal The chest was rapidly opened the lungs and the heart removed and the pulmonary artery and the left atrium were cannulated The isolated lungs were then perfused in the following way From a reservoir the blood was pumped at 25 ml min into the pulmonary artery In all experiments the pulmonary arterial pressure was between 10 and 0 mm Hg The pulmonary venous outflow pressure was kept constant at + 2–3 cm H_2O by adjusting the outlet of the left atrial cannula The blood escaped into a reservoir and was then pumped to superfuse 4 assay tissues The 4 tissues included rabbit aorta (RbA) rat colon (RC) chicken rectum (CR) and rat stomach strip (RSS) A diagram of the set up is shown in Fig 1 Changes in smooth muscle tension were measured by Harvard smooth muscle transducers and record d on a Watanabe -channel pen recorder Before being used for bioassay the tissues were bathed for 1½ h in Krebs solution gassed with 5 CO_2 and 95 O_2 and containing a combination of antagonists that eliminate the actions of histamine catecholamines serotonin and acetylcholine (Piper and Vane 1969) These were (in g/ml) hyoscine hydrobromide 10^{-} propranolol hydrochloride 2 10^{-6} methysergide bimaleate 10^{-7} and mepyramine maleate 10^{-7} Calibrating doses of PGE_2 and $PGF_{2\alpha}$ were repeatedly infused directly to the assay tissues Perfusion pressure was measured by recording the pressure in the pulmonary artery

Results

In order to induce platelet aggregation a suspension of collagen fibrils (Bo and Hognestad 1972) was infused into the pulmonary artery (1 ml over 1 min) The observation

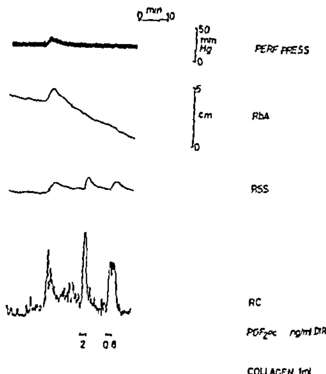


Fig 2 Pulmonary perfusion pressure and contractions of 3 assay tissues (rabbit aorta (RbA) rat stomach strip (RSS) and rat colon (RC)) subsequent to infusion of collagen into the pulmonary artery Collagen infusions increase perfusion pressure and release a rabbit aorta contracting substance (RCS) and PG like substance (s) (PG LS) PG LS in this example could be matched by calibrating doses of $PGF_{2\alpha}$

monary pressor response is a verification that platelet aggregation has been induced as collagen per se has no effect on the pulmonary circulation (Vaage Bo and Hognestad 1974) A total of 7 collagen infusions into lungs were performed in 5 expts Such infusions always caused a rise in the perfusion pressure of 10 20 mm Hg about 1–2 min after the end of each infusion and all assay tissues always contracted (Fig 2) The time course of the contraction and the rise in perfusion pressure (Fig 2) were similar This contraction was short lasting The only substances so far found to contract RC RSS and CR simultaneously in the presence of combined antagonists are PGs of the E and F series (Piper and Vane 1969 1971) Therefore contraction of this system showed release of some PG like substance(s) (PG LS) during platelet aggregation induced by collagen The contraction of RbA showed release of a rabbit aorta contracting substance (RCS) which may be a substance intermediate in the biosynthesis of PGs (Gryglewski and Vane 1971) possibly an unstable endoperoxide (Hamberg and Samuelsson 1973)

In each of the 5 expts an additional infusion of collagen was made with the lungs replaced in the circuit by a blood infusion filter with a pore size of 40 μm (Bentley Polyfilter PF 127 Bentley Lab Inc) This was done either initially or at the end of the experiment The contractions of assay tissues elicited by infusion of collagen into the lungs or filter were compared with those caused by calibrating doses of PGs given directly to the assay tissues When the filter was used the addition of collagen caused a contraction of the assay tissues which both quantitatively and qualitatively was similar to the contractions seen after collagen infusion into lungs

The contractions of RC RSS and CR could usually not be exactly matched by calibrating infusions of either PGE_2 or $PGF_{2\alpha}$ which are released during platelet aggregation (Smith *et al* 1973) but combinations of these PGs might have caused the contractions in all tissues except RbA When the active material released was assayed as PGE_2 or $PGF_{2\alpha}$ it appeared that amounts of 1–5 ng/ml were released Fig 2 shows one expt where the contractions could be matched by calibrating doses of $PGF_{2\alpha}$ The concentrations of PGs used for calibration of the assay tissues did not however have any effect on the pulmonary perfusion pressure when given i a Neither did such infusions cause any contraction of the assay tissues indicating effective inactivation in the lungs

As a control the addition of collagen to the assay tissues was tested when they were superfused with Krebs solution instead of blood No contraction of the assay tissues was then elicited Neither did histamine nor serotonin doses of 0 5–1 μg/ml given directly to the assay tissues cause any contractions

Discussion

One important consequence of induced platelet aggregation in the circulation is smooth muscle constriction in the airways and in the lung vessels supposedly due to humoral factors released from platelets (Rådegran and McAslan 1972 Vaage Bø and Hognestad 1974 Vaage *et al* 1974) This pressor response was also observed in the present experiments in spite of lung perfusion with a very low flow The amount of PG LS detected in blood was small and equivalent concentrations infused did not cause any pulmonary vasoconstriction However the concentration locally at the site of the trapped platelet microemboli close to the smooth muscle cells might be far higher $PGF_{2\alpha}$ constricts both bronchial and pulmonary vascular smooth muscles (Änggård and Bergstrom 1963 Sweatman and Collier 1968) RCS contracts isolated vascular and bronchial smooth muscle but its action on blood vessels and airways *in vivo* is unknown (Piper and Vane 1969 Piper and Walker 1973) Consequently PGs and RCS might be direct mediators of at least part of the pulmonary pressor response during platelet embolism This suggestion is supported by the observation that the time course of PG LS release is parallel to the time course of the pulmonary pressor response Alternatively PGs might modulate and even enhance the actions on smooth muscle of other possible mediators such as for instance 5 hydroxytryptamine as they are known to modulate vascular smooth muscle reactivity (Greenberg *et al* 1973 Greenberg and Long 1973)

By exchanging the lungs with a filter to trap platelets we hoped to determine quantitatively how the lungs could modify the amount of PG LS and RCS in blood after intravascular platelet aggregation Theoretically release from the lungs themselves and/or inactivation could take place PGs infused into lungs are rapidly inactivated in the pulmonary circulation (Ferreira and Vane 1967 Piper Vane and Wyllie 1970) and PG release from lungs is induced by a variety of mechanical and chemical stimuli (Piper and Vane 1971) However in the present experiments the same amount of active materials was detected whether lungs or a mechanical filter was in the circuit This could be achieved by inactivation of PGs released from platelets at the same time as PGs are released from the lung tissue It seems

unlikely, however, that the lungs would release PGs to exactly compensate for the amount which was inactivated.

Another explanation of our findings is that no PGs are released from lungs. Indeed the platelet aggregates probably have different mechanical properties from the various non-biological emboli used in other investigations. PGs released from lungs in these investigations might also be due to secondary changes and not to the emboli per se. PGs released from lungs by anaphylaxis are not available to the unactivation mechanism in the pulmonary circulation (Piper 1974) and PGs released from platelets may also not be inactivated in the pulmonary vascular bed. This could easily happen if PG release occurs downstream to this mechanism. In the present experiments there is no reason to question the capacity of the pulmonary inactivation mechanism, which at least was able to remove PGs in the concentrations detected during release, when PGs were infused into the pulmonary artery.

In conclusion, PGs and RCS are released in parallel to the pulmonary pressor response during pulmonary microembolism due to intravascular platelet aggregation. However, as the presence of lungs in the circuit did not modify the amount of PGs detected after induced platelet aggregation, it seems possible that PGs and RCS are released from platelets only and not from lungs as well. These vasoactive substances might be involved in the vascular and bronchial smooth muscle constriction observed in the lungs after induced intravascular platelet aggregation.

We thank Mr. M. A. Palmer for skilled technical assistance. JV thanks the Norwegian Research Council for Science and the Humanities for a Research Fellowship and for additional support in connection with experiments carried out at the Royal College of Surgeons of England. PJP thanks the Medical Research Council for a grant.

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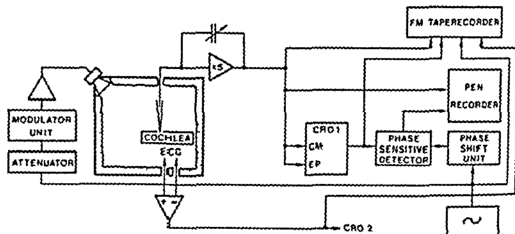


Fig. 1 Experimental arrangement for recordings of the endolymphatic potential (EP) and the cochlear microphonic potential (CM) from the pigeon with a microelectrode The recorded signals from the cochlea were processed as shown by the block diagram The stimulus tones were delivered from a loudspeaker placed in the ceiling of a sound attenuating box The ECG was recorded and displayed on an oscilloscope (CRO 2) For more detailed description see Methods

was artificially respirated through a silicone rubber tube placed in the trachea (30 strokes min) By a three way stopcock the pigeon could be respirated with air or nitrogen

During the experiment the pigeon was covered by cotton wool to minimize heat loss The rectal temperature was found to decrease about 1 C/h after administration of Tubocurarin chloride The total duration of the experiments was usually 2 3 h

Surgical p ocedu e

When a satisfactory level of anesthesia had been achieved an incision was made posterior to the opening of the ear canal The muscles were carefully dissected away with minimal blood loss until the bone of the skull was uncovered The wall of the skull was opened by a dental drill (air driven) and the bone trabeculae between the skull and the labyrinth removed with watch maker s forceps leaving a clear view to the proximal p t of the cochlea The cochlear wall was carefully thinned with a fine burr close to the window region. The d ll ng was topped when the connecti e tissue (periost) of the inner wall of scala vestibuli was reached. This t s ue was perforated with a sharpened needle

Anat m

The gross anat my and histology of the as an cochlea have been described by Retzius (1884) A more detailed ac ount of the distribution of the differentiated hair cells along the papilla basilaris and the innervation pattern f the hair cells has been gi en by Takasaka and Smith (1971)

Elect od

Highly cleaned glas tubes (Corning, code 7740) were drawn on a horizontal electrode puller The ti diameter measured b lect on microscopy was 0 18–0 . μm The tips of the p pettes were f lled th distilled water (Zeuth n 19) and the haft with 3 M KCl through a hypodermic needle The p pettes were then placed in a b ttle and mmersed n membrane filtered 3 M KCl (pore size of the filter 0 1 μm) Before the pipettes were mounted n an electrode holder with a Ag AgCl electrode the electrolyte of the shaft was replaced by a 0 15 M KCl s lut on Electrodes prepared in this way had a resistance of 10 70 MΩ measured in Ring r s lut n and a t p potential of less than 5 mV

Registrati n

The measuring circuit is shown in diagrammatic form in Fig. 1 The pigeon was grounded via a sa re bridge (0 15 M KCl in 3 Ag r) between undamaged muscles in the neck and an Ag AgCl electrode The microelectrode was mounted in a mod f ed micromanipulator by which the electrode c uld be ad

vanced in steps of 0 8 μm by a stepmotor (Slo-Syn The Superior Electric Company Bristol Connecticut U S A) The microelectrode was connected to the input of a preamplifier (Bioelectric, Negative Capacitance Amplifier NF I) and the output from this stage representing the potential difference between the microelectrode and ground was fed to the input of an oscilloscope (Tektronix 502 A) a potentiometer pen recorder (Servogor) and a FM tape recorder (Philips Analog 7) The output from the preamplifier which also contained an ac signal (CM) when the ear was stimulated with sound was fed into the second channel of the oscilloscope (ac-coupled) After suitable amplification the signal was led to a Phase Sensitive Detector (Brookdeal 411) and to a tape recorder A sine voltage from a function generator (Wavetek) served as reference signal for the phase sensitive detector The sine voltage was frequency and phase locked to the tone generator (Hewlett Packard 200 CD) which governed the sound stimulus The phase of the reference signal could be varied continuously from 0 to 360 The output of the phase sensitive detector was recorded on the second channel of the potentiometer recorder This output signal represented the amplitude of CM when the phase angle between CM and the reference signal was 0 or 180

Sound stimulus

The output of the tone generator (HP 00 CD) was fed to a modulator unit where the pulse durating, on off rise times of the pulse and repetition rate could be selected These signals were fed to a power amplifier which was connected to a loudspeaker placed in the ceiling of a sound attenuating experimental box The voltage to the loudspeaker was cal brated to give a sound pressure level (SPL) of 70 dB (re 0 0002 dyn cm) at the site of the pigeon s head When different SPL s were used the voltage to the loudspeaker was attenuated in 5 dB steps The maximal SPL used in these experiments was 95 dB The calibration procedure was performed with a 1/8 condenser microphone (Bruel & Kjær type 4138) connected to a 1/3 octave filter (Bru l & Kær type 1612) and a m crophone amplifier (Bruel & Kjær type 606) All frequenc es used during the experiments were calibrated at regular intervals

Elect ode re stan e

In order to check the integrity of the electrode tip the electrode resistance was measured at regular intervals during the experiments An intermittent triangular voltage was differentiated by a small capacitor connected to the preamplifier input The diphasic rectangular current pulses flowing through the electrode to the ground generated an input voltage to the preampl fier the amplitude of which was proportional to the total resistance to ground (Lettvin *et al* 1958)

Analy s of th cochl or m rophon e potential (CM)

When the electrode was in ductus cochlearis the stray capacitance of the recording system was maximally neutralized by negative capacitance feed back giving a time constant for the input of the record ng system of less than 0 2 msec This procedure increased the noise of the record ng system and consequently small amplitudes of CM were buried in noise For analysis of the amplitude of CM during anoxia the s gnal was fed to a band pass f lter (Bruel & Kjær type 161?) and a microphone amplifier (Bruel & Kjær type 606) The r m s value of the signal from the amplif er was recorded on the potent om ter recorder In two experiments the signal to-noise ratio was increased by break ng the electrode t p Thus it was poss ble to investigate the wave form of CM

The relati e phase variation of CM during anoxia was analyzed using a Phase Sen t Detector (PSD) The dc voltage from the PSD is p oportional to the voltage of the s gnal times the cos ne of the phase angle between the signal and the reference voltage Thus wh n gn l and fer nce oltages wer 90 out of phase the dc signal from the PSD was zero and ndependent of amplitude hang of the signal voltage Changes in the phase angle of CM du ing anox a ga e a d signal from the PSD d fferent f om z ro S nce the voltage is dependent upont the change n phase and th actual amplitud of CM the elati e phase angle of CM could be cal ulated Th t m con tant of th PSD was u ually set at 300 ms g v ng a bandwidth of approximately 0 1 Hz (bandw dth (Hz) = 1 3 (t me constant (s)))

Expe men al p o edu

The mic oelect ode was placed n the scala vestibuli close to the columella footplat and ad anced w th a constant step fr qu ncy towa ds the papilla ba ila is w thout v sual control of the electrode tip Both the dc potent al and th ampl tude of the fundamental f equency of CM were eco ded upon the pot nt omete recorde th electrode wa ad anced th ough the cochlea The electrod t p was as umed to be in the ductus cochle when th amplitud of CM increased s multaneously with ecord ng of a pos ti

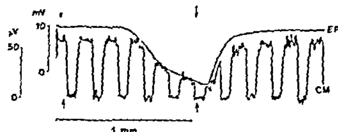


Fig. -. The effect of transient anoxia on the endolymphatic potential (EP) and the amplitude of CM. The upper trace shows the change in EP during transient anoxia. The lower trace shows the r.m.s. value of the fundamental frequency of CM measured by the microphone amplifier. The ear was stimulated with 4 kHz sound pulses of eight seconds duration at four second intervals. Nitrogen breathing was initiated at the time indicated by the first arrows and terminated 60 s later indicated by the next pair of arrows (the 2 curves are displaced horizontally due to the pen recorder arrangement). The best frequency in this experiment was 4 kHz.

and wi h orl a minor associated phase shift (0 -30) Furthermore this positi e potential should be sensitive to anoxia.

S nce the exact position of the electrode in the ductus cochlearis could not be determined CM frequency response curves were obtained in each animal at 70 dB SPL with the electrode tip in ductus cochlearis. This functional measure of position relative to the papilla basilaris served to compare the results from different pigeons.

The anoxia experiments were performed with the electrode in ductus cochlearis. The pigeons were made anoxic by entilating with pure nitrogen for 40-90 s. The pigeons were always reventilated with air before brad cardia occurred. During the ent re period both EP and CM were recorded. When th amplitude changes of CM at different frequencies were studied successive periods of anoxia were conducted with 5 10 min intervals between them. In each p geon the change in CM at one or more frequencies duri g transient anoxia was in estigated at least twice during the course of the experiment. The observed values CM changes during anoxia were not significantly different indicating that successi e periods of anoxia did not affect the demonstrated frequency-dependent change of CM amplitude during anoxia.

Results

General pattern of changes

The effect of transient anoxia upon CM at different frequencies at constant sound pressure level (70 dB) was studied in 22 pigeons. In 9 pigeons the effect of transient anoxia upon CM at different frequencies and at varied sound pressure level was investigated. The effect on both the amplitude and the phase angle was examined in all experiments.

Fig. 2 shows an example of the effect of transient anoxia upon EP and the r.m.s. value of CM at 4 kHz. The reduction of CM began 27 s after anoxia was initiated. The corresponding delay for EP was 25 s. The minimum value of CM and EP was reached 8 seconds after readmission of atmospheric air. The recovery of CM and EP followed a more rapid time course than the reduction time course.

In all experiments corresponding values of EP and CM during anoxia were obtained. Since EP is known to be extremely sensitive to anoxia (Konishi, Butler and Fernandez 1961), the duration of anoxia in each experiment was determined by the time to reduce EP by 10-15 mV. This procedure gave a relatively constant change of the electrical potential gradient across the apical pole of the hair cells. The anoxia period varied between 40 and 90 s, but in all experiments with transient anoxia the change in EP was reversible.

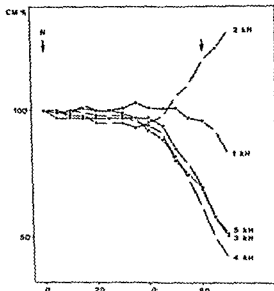


Fig. 3 An example of the effect of transient anoxia upon the amplitude of CM at different frequencies but at constant sound pressure level (70 dB) The amplitude of CM is expressed in per cent of the initial value before nitrogen ventilation Only the amplitude changes of CM during the anoxic period are shown but CM at all frequencies used returned to the initial level after air was readministrated The frequencies used are indicated at the end of the respective curves

In each experiment the electrode tip was kept in position in the ductus cochlearis At this position the effect of transient anoxia upon CM at different frequencies and EP was examined An example of the effect of transient anoxia upon CM at different frequencies is shown in Fig 3 The amplitude of CM in per cent of the value before anoxia is plotted as a function of time (seconds) during the anoxic period The last point indicated for CM at all frequencies coincides with the minimum value of EP EP in these experiments was reduced by 11 mV and the time period from the beginning of reduction to the beginning of recovery (Δt) was 43 6 ± 2 3 s CM at 3 4 and 5 kHz were found to be markedly reduced during the anoxic period and the time courses followed by CM at 3 4 and 5 kHz were almost identical In contrast to the behaviour at these frequencies CM at 2 kHz increased during anoxia After initiation of anoxia the amplitude of CM at 2 kHz was slightly reduced (3–7%) At the time when the amplitude reduction of CM at higher frequencies was accelerated CM at 2 kHz increased and continued to do so during the anoxia period The last point indicated represents the maximum value and the amplitude returned to the initial level after a slight undershoot of 10 % CM at 1 kHz was less affected by anoxia The time interval between initiation of anoxia and the reduction in amplitude of CM was larger than at other frequencies and the total reduction in amplitude was only 18% of the initial level

The CM frequency response curve (tuning curve) for a given position of the electrode tip in ductus cochlearis is shown in Fig 4 The amplitude of CM in μV r m s is plotted as a function of the frequency of the sound stimulus at constant sound pressure level (70 dB) The effect of transient anoxia is a change in the frequency response curve in such a way that the CM tuning curve becomes more flat In experiments where the low frequency slope of the frequency response curve was less steep than in the example shown the effect of transient anoxia was a change in the best frequency (the frequency at which CM had maximum amplitude) towards a lower frequency The CM frequency response curve in Fig

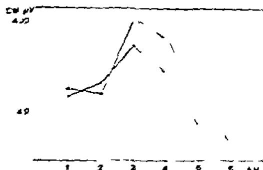


Fig. 4. The effect of anoxia on the CM frequency response curve. The amplitude of CM is plotted as a function of the frequency in a semilogarithmic scale. The amplitude of CM before anoxia is indicated by filled circles and the amplitude of CM attained after 70 s of anoxia (see Fig. 3) is indicated by open circles.

not corrected for the middle-ear transfer function. However, since the pigeon was immobilized with tubocurarine this function remains unaltered during anoxia, and the change in tuning characteristics of CM is caused by anoxia alone.

Amplitude changes of CM

The results of 115 expts. from 22 pigeons are shown in Table I. The results are divided into 2 groups depending upon the best frequency of the preparation as determined from the CM frequency-response curve obtained at 70 dB SPL.

Table I shows the calculated mean relative amplitude of CM at different frequencies after a period of anoxia. The amplitude of CM was measured at the time when the corresponding EP reached its minimum value, and the change in amplitude is expressed in per cent of the initial value.

The results show that when 3 kHz was the best frequency the average amplitude reduction of CM was largest at 3, 4 and 5 kHz. CM at frequencies less than 3 kHz were less reduced in amplitude. CM at 2 kHz in this group was either enlarged or slightly reduced in amplitude during transient anoxia. An initial enlargement in amplitude of CM during transient anoxia was found in most experiments at this frequency, but in only 36% of the experiments the amplitude continued to increase during the anoxia period.

When 4 kHz was the best frequency, the mean reduction in amplitude was largest for CM at 4 and 5 kHz. CM at 3 kHz was less reduced in this group and sometimes showed an amplitude increase followed by a slight reduction. A continuous enlargement in amplitude of CM at 2 kHz was found in 40% of the experiments; in the rest the amplitude was un-

Table I. The relative amplitude of CM at different frequencies during transient anoxia. The results from 22 pigeons are divided into groups depending upon the best frequency of each preparation. Mean values, S.E. [illegible] given for each frequency

CM (kHz)	Best frequency 3 kHz		Best frequency 4 kHz	
	CM	S.E.	CM	S.E.
CM (1)	[illegible]	[illegible]	[illegible]	[illegible]
CM (2)	[illegible]	[illegible]	[illegible]	[illegible]
CM (3)	[illegible]	[illegible]	[illegible]	[illegible]
CM (4)	[illegible]	[illegible]	[illegible]	[illegible]
CM (5)	[illegible]	[illegible]	[illegible]	[illegible]

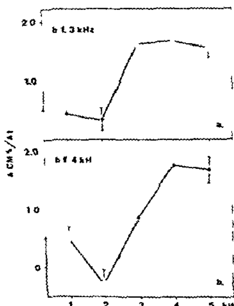


Fig. 5 The sensitivity of CM to transient anoxia, expressed as the maximal change in CM in percent of the initial level divided by the corresponding time period in seconds when the ear was anoxic as judged by the reduction in EP ($\Delta CM^{*}/\Delta t$) is plotted as a function of the stimulus frequency The sound pressure level was kept constant at 70 dB Each point represents a mean value and the numbers of observations are shown in Table I The standard errors of means are indicated by vertical bars

Fig 5 a shows the relation between the sensitivity of CM to transient anoxia and the sound frequency when 3 kHz was the best frequency (maximum amplitude of CM at this frequency) Fig. 5 b shows the relation between the sensitivity of CM and the frequency when 4 kHz was the best frequency

affected or slightly reduced during transient anoxia. CM at 1 kHz was like the first group showing only slight reduction in amplitude or no amplitude change at all during the test period

Sensitivity to anoxia

There is a total reduction of CM at all frequencies in prolonged anoxia (2–4 min) The amplitude change of CM is a function of both the time after initiation of anoxia and the frequency of the sound stimulus Therefore a better estimate of the frequency-dependent effect of anoxia on CM than the one given in Table I is needed Such an estimate may be obtained by dividing the relative amplitude change of CM by the time period of anoxia, as judged by the behaviour of EP This time period (Δt) was in each experiment taken to be the time between the start of the decrease of EP and the start of the increase in EP after air was readministrated (see Fig 2)

In Fig 5 a and b the mean sensitivity of CM to anoxia, ($\Delta CM_{\%}/\Delta t$) is plotted as a function of the frequency of the sound stimulus The sensitivity of CM at 3 4 and 5 kHz was higher than at 1 and 2 kHz when 3 kHz was the best frequency When the best frequency of the preparation was 4 kHz the pattern was different The sensitivity of CM at 4 and 5 kHz was larger than at 1 2 and 3 kHz. The mean sensitivity of CM at 4 kHz was found to be significantly different from the mean sensitivity of CM at 3 kHz ($P < 0.001$)

Comparison between the results of Fig 5 a and 5 b shows that the mean sensitivities of CM at 1 4 and 5 kHz to anoxia were alike At 2 and 3 kHz the sensitivity of CM to anoxia was lower when the best frequency was 4 kHz than when 3 kHz was the best frequency The difference in sensitivity to anoxia at these frequencies was found to be significant ($P < 0.001$) Thus the sensitivity of CM to transient anoxia was found to be dependent upon the frequency as well as the best frequency of the preparation. CM at frequencies equal to

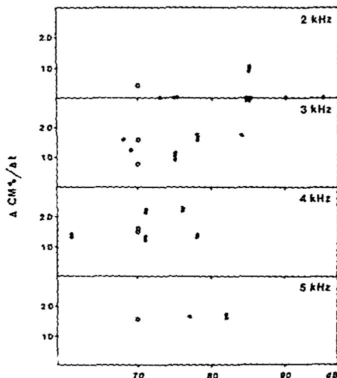


Fig 6 The sensitivity (ΔCM / Δt) of CM to transient anoxia is plotted as a function of the sound pressure level (dB) The frequency of the sound stimulus is indicated Each point represents one experiment Open circles represent values of the sensitivity of CM taken from Fig. 5

and higher than the best frequency was more sensitive to transient anoxia than CM at frequencies lower than the best frequency CM at frequencies 1 kHz lower than the best frequency often showed a negative sensitivity (corresponding to an increase in amplitude) during transient anoxia

Effects of intensity differences

The above described results were obtained at constant sound pressure level (70 dB) Thus at this point it is uncertain whether the observed differential effect of anoxia is a frequency dependent effect or an effect of the difference in absolute amplitude of CM at the used frequencies

In a separate series of experiments the influence of varied sound pressure level upon the CM sensitivity to anoxia was examined The sensitivity of CM to anoxia in each experiment was calculated and plotted as a function of the sound pressure level (Fig. 6) for various frequencies of the sound stimulus.

From the distributions of observations for CM at 3 4 and 5 kHz, the sensitivity of CM to anoxia seems to be unrelated to the sound pressure level For each frequency the regression line for the observations was calculated None of the slopes of the lines was significantly different from zero In experiments where CM at 2 kHz was enlarged in amplitude during anoxia at low SPL, elevation of SPL was found to reduce the enlargement or even cause a minor reduction The tendency observed in many experiments at frequencies different from 2 kHz, that elevation of SPL reduced the sensitivity could not be shown to be significant either

TABLE II The difference between the time periods for reduction in amplitude (CM) and potential (EP) during transient anoxia at different sound frequencies Mean values ± S E. and number of experiments

CM (kHz)	t(CM EP) ± S E. seconds	n
CM (1)	8 ± 5 0	13
CM (?)	30 ± 3 5	1
CM (3)	± 0 9	—
CM (4)	7 ± 4 4	13
CM (3)	18 ± 1 7	1
CM (4)	± 0 9	18
CM (5)	2 ± 0 9	11

The frequency in the bracket is the best frequency

The relation between the time courses of CM and EP

The time course of the changes in CM was compared to the corresponding changes in EP during transient anoxia The time period from the beginning of nitrogen breathing to the first perceptible reduction in CM and EP was recorded (cf Fig. 2) and the difference was calculated Table II shows the results

The reductions in CM at 1 and 2 kHz were markedly delayed relative to that in EP The delay at 3 kHz was not significantly different from zero when 3 kHz was the best frequency but CM at 3 kHz was delayed relative to EP when 4 kHz was the best frequency The values for CM at 4 and 5 kHz were not significantly different from zero

The changes in amplitude of CM and in EP were examined at different times during the anoxia period and correlation coefficients between the relative changes in corresponding values of CM and EP were calculated When the frequency was equal to and higher than the best frequency very high correlation coefficients were found ranging between 0 91 and 0 99 in 26 expts

Changes in waveform of CM

The above given description of amplitude changes of CM during transient anoxia is restricted to the r m.s value of the fundamental frequency of the electrical signal from the cochlea The influence of anoxia upon the waveform of CM was examined less systematically In two experiments the electrode tip was broken so that larger signal to noise ratios were obtained thereby allowing for visual examination of the waveform

From the visual examination the waveform of CM was not changed during transient anoxia During prolonged anoxia the amplitude of CM at 2 kHz was reduced and the wave form of CM was markedly distorted This is in contrast to the change in CM at 3 kHz where a distortion of the waveform could not be observed. In these experiments 3 kHz was the best frequency The distortion of CM at 2 kHz during prolonged anoxia is shown in Fig. 7 The effect of anoxia is seen to be complex. Besides a change in waveform anoxia caused a change in the phase angle of CM relative to the initial phase (see below)

The relative phase angle of CM

Anoxia is expected to affect only the mechanisms responsible for EP and eventually the hair cell potential The differential effect of anoxia upon the amplitude of CM indicates

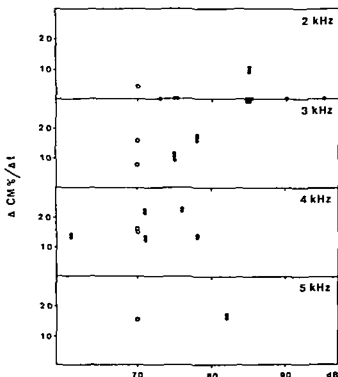


Fig. 6. The sensitivity (ΔCM / Δt) of CM to transient anoxia is plotted as a function of the sound pressure level (dB). The frequency of the sound stimulus is indicated. Each point represents one experiment. Open circles represent values of the sensitivity of CM taken from Fig. 5.

and higher than the best frequency was more sensitive to transient anoxia than CM at frequencies lower than the best frequency. CM at frequencies 1 kHz lower than the best frequency often showed a negative sensitivity (corresponding to an increase in amplitude) during transient anoxia.

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From the distributions of observations for CM at 3, 4 and 5 kHz, the sensitivity of CM to anoxia seems to be unrelated to the sound pressure level. For each frequency the regression line for the observations was calculated. None of the slopes of the lines was significantly different from zero. In experiments where CM at 2 kHz was enlarged in amplitude during anoxia at low SPL, elevation of SPL was found to reduce the enlargement or even cause a minor reduction. The tendency observed in many experiments at frequencies different from 2 kHz, that elevation of SPL reduced the sensitivity could not be shown to be significant either.

The maximum value of the phase shift calculated (Fig. 8 b) corresponds to the value obtained from direct reading on the oscilloscope (Fig. 8 c)

Changes of the phase angle of CM during transient anoxia were investigated in the anoxia experiments described above The changes in phase of CM were found to be a complicated function of stimulus frequency and the sound intensity The largest changes in phase were found around the best frequency

Discussion

The results of this study show that the effect of transient anoxia on the amplitude changes of CM at constant sound pressure level is dependent upon the frequency of the sound stimulus When the sound pressure level and thereby the amplitude of CM was changed the differential effect of transient anoxia remained unaltered This fact excludes the possibility that the demonstrated differential effect of anoxia upon the amplitude changes of CM is due to the difference in amplitude of CM at different frequencies when the sound pressure level is constant Thus it can be concluded that transient anoxia produces a frequency-dependent change in the amplitude of CM

As a relative measure of the position of the electrode tip in ductus cochlearis the best frequency was determined from the CM frequency response curve obtained at constant sound pressure level The best frequency has been shown to change with the position of the electrode tip along the basilar membrane (Honrubia and Ward 1968) The best frequency in all experiments in this study was either 3 or 4 kHz, indicating that the electrode tip was located mainly in two positions in the ductus cochlearis

The division of the results into two groups depending upon the best frequency of each preparation gives two anoxia sensitivity curves (sensitivity of CM as a function of the frequency Fig 5) They both show that CM at frequencies equal to and higher than the best frequency is more sensitive to transient anoxia than CM at frequencies lower than the best frequency The findings that CM at 3 kHz sometimes behaves like low frequency CM and sometimes like high frequency CM appears to be related to the best frequency of the preparation When CM at 3 kHz is the best frequency the sensitivity of CM at this frequency to transient anoxia is high The relation between the sensitivity of CM to transient anoxia and the best frequency suggests that the effect of anoxia is a positional one The difficulties in gaining access to more distal parts of the ductus cochlearis with a microelectrode prevented further verification of this suggested positional effect of anoxia

Using differential electrodes placed along the basilar membrane in guinea pig Dallos (1973 b) recently demonstrated that the 3 dB cut-off frequency of the CM frequency response curve at low SPL (50 dB) corresponded to the frequency which produced maximal vibration of the basilar membrane at that location CM frequency response curves recorded with a single electrode in ductus cochlearis of the pigeon at low SPL resemble CM frequency response curves from the guinea pig recorded with a differential electrode technique They are similar with respect to non linearity of the best frequency and the high and low frequency slope (Jorgensen in prep) This similarity in CM frequency response curves from the guinea pig and the pigeon suggests that CM recorded with a single electrode in d

chlearis does reflect the displacement pattern of the basilar membrane better than previously believed

A consequence of the frequency dependent change of CM is that transient anoxia produces changes in the CM frequency response curve (cf Fig 4) This curve becomes flattened and the best frequency may be shifted towards a lower frequency The shift is a consequence of the difference in sensitivity of CM to transient anoxia but may be emphasized by an increase in amplitude of CM at 1 kHz lower than the best frequency

Although the mechanism for the frequency dependent change in amplitude of CM during transient anoxia is not known the finding that the sensitivity of CM at different frequencies is related to the best frequency rather than the numerical value of the stimulus frequency indicates that the effect of transient anoxia is positional The change in the CM frequency response curve produced by transient anoxia suggests that changes in the mechanical tuning of the basilar membrane take place

Transient anoxia was formerly believed to influence the endolymphatic potential only but recent investigations of the vibration pattern of the basilar membrane of the squirrel monkey during death showed that the tuning of the basilar membrane is physiologically vulnerable (Rhode 1973) A decrease in amplitude of vibration by a factor of 10 over a 10 min period immediately after death was observed The first and most marked chances occurred for frequencies near and greater than the frequency which produced maximal vibration of the area of the basilar membrane under observation This region of the basilar membrane attained a lower frequency as the best frequency but the non linear behaviour of this region (Rhode 1971) disappeared These changes in the tuning characteristics of the basilar membrane during death were accompanied by changes in the phase of the vibration relative to the stapes The changes in pattern of vibration of the basilar membrane may indicate that both an increase in damping and a decrease in stiffness of the basilar membrane take place during death

Recently Evans (1973) and Klinke and Evans (1973) demonstrated that hypoxia and metabolic inhibitors caused changes in the frequency threshold curves of single cochlear fibres in the guinea pig The low threshold sharply tuned curves became broadly tuned high threshold curves In some cases the characteristic frequency was shifted towards a lower frequency when metabolic inhibitors were administrated These findings were interpreted as a loss of a second filter subsequent to that of the basilar membrane This filter has been proposed to account for the difference in tuning between the basilar membrane and the single cochlear fibre The finding however may also indicate that hypoxia and metabolic inhibitors cause changes in the vibration pattern of the cochlear partition

When the changes in vibration pattern of the basilar membrane of the squirrel monkey during death are compared with the frequency-dependent changes in amplitude of CM in the pigeon during transient anoxia the striking qualitative resemblence is noted Although the interpretation of the CM amplitude changes is dependent upon more precise knowledge of the quantitative relation between extracellular recorded CM and the corresponding vibration of the basilar membrane underneath the electrode tip the results give evidence that transient anoxia causes similar changes in the vibration pattern of the basilar membrane in the pigeon ear This would mean that the amplitude changes of CM during anoxia were

caused by the combined effect of changes in the endolymphatic potential and changes in the mechanical vibration pattern of the cochlear partition The demonstrated changes in the relative phase angle of CM during transient anoxia with major changes around the best frequency are in agreement with this concept

In search for the mechanism responsible for the changes in the vibration pattern of the cochlear partition during anoxia the close coupling in time between the reduction in the endolymphatic potential and the amplitude reduction of CM at frequencies equal to and higher than the best frequency may be of significance Since only a part of the amplitude change of CM can be accounted for by the change in the electrical potential gradient (Davis model) the remaining change of CM may be caused by the suggested changes in pattern of vibration which then must follow a time course similar to that of EP The findings of Klinke and Evans (1974) may support this notion since metabolic inhibitors most likely have the largest effect on the endolymphatic potential Changes in the hydrostatic pressure in ductus cochlearis may be the relevant change since Tonndorf (1957) demonstrated that an increase in volume of the endolymphatic space in a cochlear model caused a reduction in the amplitude of the basilar membrane vibration The reduction was found to be frequency dependent with largest amplitude reduction in the distal end of the cochlea (lower fre quencies) Furthermore a shift in locus of maximum displacement amplitude for a particular frequency towards the proximal end was observed

Recently Necker (1970) reported that transient anoxia produces complex changes in microphonic potential in pigeon starling and sparrow CM was elicited by acoustic clicks (rarefaction and condensation) and recorded with electrodes in the perilymphatic space The results have been interpreted in terms of the hair cell transducer mechanism and do not appear to offer an explanation for the phenomenum investigated in this study

In conclusion it has been shown that transient anoxia causes a frequency-dependent change of the amplitude of CM The results suggest this effect to be a positional one such that CM at frequencies equal to and higher than the best frequency is more sensitive to anoxia than CM at frequencies lower than the best frequency CM at 1 kHz lower than the best frequency often increases in amplitude during transient anoxia It has been proposed that the frequency dependent change in CM amplitude is a consequence of changes in the vibration mode of the basilar membrane caused by anoxia Thus the amplitude changes of CM during anoxia are caused by the combined effect of changes in EP (the electrical potential gradient across the apical pole of the hair cells) and changes in the pattern of vibration in the co- chlear partition The observation that changes in the amplitude of CM are associated with changes in the relative phase angle of CM supports this hypothesis

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Acta physiol scand 1975 94 29–35

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Transcapillary Fluid Movements in Sympathectomized Intestine and Skin during Hemorrhagic Hypotension

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Received 14 November 1974

Abstract

JARHULT J and P O GRANDE *Transcapillary fluid movements in sympathectomi ed in testine and skin during hemorrhagic hypotension* Acta physiol scand 1975 94 29–35

Net transcapillary fluid exchange in skin t ssue (paw) and small intest ne was observed during a 90 min period of hemorrhag c hypotension at 50 mm Hg in the cat Reflex fluid transfer was prevented by regional sympathectomy and α adrenergic blockade Early in hemorrhage fluid absorption from the extravascular space occurred in both tissues apparently caused by osmosis The process was thus co-ord nated in time with a positive arterio-venous osmolar difference in turn caused by a marked arterial hyperosmolality Experimental arterial hypero molality of similar magnitude created by i v infusion of hypertonic gluco e in non bled animals led to transcapillary fluid absorption in both intestine and skin and at rates similar to those in bleeding Regional hypotsionen pe e caused no flu d absorption Later in hemorrhage (> 30 min) plasma fluid moved into the extravascular space both in skin and intestine apparently due to a grad ual increase of capillary hydrostatic pressure It is concluded that the arterial hyperosmolality during bleed ing can cause transcapillary fluid absorption in intestinal and skin tissues, as previously shown for skeletal muscle (Jarhult 1973) The hemodynamic signif cance of this process for plasma volume regulation in hemorrhage is however much greater in skeletal muscle than in intestine and sk n mainly due to the much larger total mass of the muscle tissue

During hemorrhagic hypovolemia extravascular fluid from skeletal muscle is mobilized into the blood stream This transcapillary fluid absorption is caused partly by an adrenergic reflex decrease of the capillary hydrostatic pressure (e g Öberg 1964) and partly by an osmotic mechanism related to arterial hyperosmolality in turn created by glucose release from the liver (Järhult 1973) The latter study indicated that the osmotic mechanism was responsible for about 50 per cent of the total extravascular fluid absorption from cat skeletal muscle during hemorrhagic hypotension at 50 mm Hg In the present investigation an attempt was made to analyse whether such an osmotic fluid transfer can occur in tissues other than skeletal muscle ei intestine and skin

Methods

The stud was perf med on cats anesthetized with α hlo alose (50 mg/kg) initially supplemented a small dose of pentoba b t l sod um (10–15 mg) The animals w re deprived of food fo 16 h exper ment

Intestinal preparation In 10 cats without obvious signs of intestinal infection an intestinal preparation was made according to that described by Folkow *et al* (1963) Briefly a segment of the jejunum (weighing 15–20 g) was isolated together with its lymph node and the remainder of the small and large intestines removed The regional autonomic nerves were ligated and cut A short arterial shunt circuit was inserted in the superior mesenteric artery The venous outflow from the segment and its lymph node was measured with an optical drop recorder and returned to the animal via the jugular vein To record changes in tissue volume the intestinal segment was enclosed in a perspex temperature controlled (38 C) plethysmograph filled with Tyrode s solution

Skin preparation The hind paw was chosen for the study of the circulatory events in a cutaneous vascular bed (12 cats) The pads (with abundant arterio-venous anastomoses) were excluded from the circulation by tight ligatures Hence the reactions observed can be considered to have occurred mainly in nutritive skin vessels The skin of the calf was dissected free from the knee down to the ankle joint All superficial and deep veins were ligated except for the great saphenous vein which was cannulated and the venous outflow diverted to the jugular vein via an optical drop recorder The paw was placed in a perspex plethysmograph filled with water using the calf skin flap for proximal closure The temperature was kept at 34 C (≈ neutral temperature) In 2 expts the circulatory events in the paw were studied during regional hypotension For this purpose a short shunt circuit was inserted between the femoral and popliteal artery all other arterial vessels between the thigh and the calf being ligated Regional arterial pressure was then monitored from a T tube in the shunt and could be adjusted to desired levels by a screw clamp The weight of the different tissues in the paw was determined after each experiment On the average skin was found to comprise 26 per cent tendons fat and muscle 21 per cent and bone 53 per cent (in agreement with Öberg 1964)

General experimental procedure After heparinization the right carotid artery was cannulated and connected to a siliconized pressure bottle to permit bleeding Mean arterial blood pressure was monitored from the left femoral artery and measured with a Statham P23 AC transducer The regional α adrenoceptors in the intestine and skin were blocked with a close arterial injection of phenoxybenzamine (1 3 mg) To avoid systemic effects of this drug, the venous outflow from the region was collected for 2 3 min after the injection discarded and substituted by the same amount of dextran The effectiveness of the α adrenoceptor blockade was checked by close arterial injections of noradrenaline

Changes of regional vascular resistance were expressed as per cent of control resistance before bleeding Changes of tissue volume were continuously recorded with a Grass FT 10C transducer connected to the plethysmograph With this technique a rapid change of tissue volume after bleeding represents the capacitance response and later slower changes represent transcapillary fluid movements (for details see Grände *et al* 1974) Plasma osmolality was analysed with thermistor cryoscopy (Adv Instruments Inc) Spread of data is given as S E

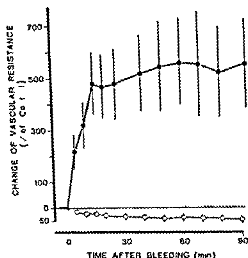
Protocol The animals breathed spontaneously and were permitted to recover for 30 min after the completion of the surgery Venous outflow pressure in the intestine and the skin was adjusted so as to accomplish an isovolumetric state (transcapillary fluid equilibrium) in the control period Late in this period blood samples were withdrawn from the carotid artery and from the superior mesenteric or great saphenous vein for determinations of control plasma osmolality The animal was then bled rapidly (< 5 min) to mean arterial pressure of 50 mm Hg a level maintained throughout the experiment by means of the pressure bottle Samples for arterial and venous plasma osmolality determinations were taken 5 10 15 20 30 40 50 70 and 90 min after the start of the bleeding

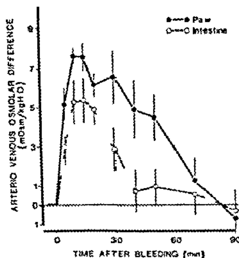
Results

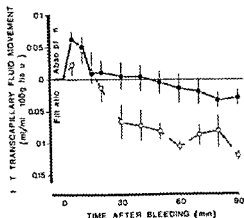
Intestine

The control data before hemorrhage in the sympathectomized α blocked small intestine (8 expts) were as follows Arterial inflow pressure 104 ± 6 mm Hg, venous outflow pressure 7 ± 3 mm Hg blood flow 33 4 ml min 100 g regional resistance 3 0 0 7 mm Hg (ml min × 100 g) arterial plasma osmolality 320 ± 3 and venous (superior mesenteric) plasma osmolality 322 ± 2 mOsm/kg H O

Hemorrhage to an arterial pressure of 50 mm Hg caused a marked gradual decrease of the intestinal vascular resistance (Fig 1 panel A) after 30 min of hypotension it was re







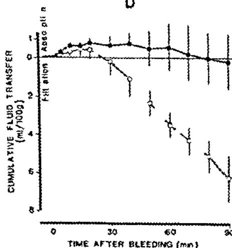


Fig. 1 Circulatory events in the sympathectomized α blocked paw (●—● 8 expts) and small intestine (O O 8 expts) during hemorrhagic hypotension at 50 mm Hg in the cat Panel A show the change of vascular resistance panel B the arterio-venous osmolar difference panel C the net transcapillary fluid movement and panel D the cumulative capillary fluid transfer Mean values ± S E are given

duced to about 50 of the control value implying that blood flow then had returned to the prehemorrhagic level Arterial plasma osmolality increased by about 20 mOsm kg H_2O above control after 20–30 min of arterial hypotension in accordance with previous findings (Jarhult 1973) A moderate positive arterio-venous osmolar difference was noted in the early (30 min) phase of hemorrhage (Fig 1 panel B) indicating that a transcapillary osmolar gradient was present in this period (see Lundvall 1972)

The concomitant net transcapillary fluid movement is depicted in Fig 1 panel C Absorption of extravascular fluid to the blood stream occurred in the initial period of hemorrhagic hypotension and at a maximal rate of 0 045 ± 0 021 ml/min × 100 g tissue The absorption process then declined and there was a reversal to net fluid filtration which after 30 min of hemorrhage and later occurred at rates of 0 07–0 12 ml/min × 100 g The fluid absorption was present at a time when the positive arterio venous osmolar difference was most pronounced suggesting that it was an osmotic process related to the hemorrhagic hyperosmolality This hypothesis was supported by experiments on non bled cats (2 expts) which were merely exposed to an arterial hyperosmolality induced by slow intravenous infusions (n = 5) of hypertonic glucose solutions Such arterial hyperosmolality (+15 to 25 mOsm/kg H_2O) regularly evoked a transcapillary fluid absorption in the α blocked small intestine which amounted to 0 05–0 1 ml/min × 100 g tissue

The cumulative capillary fluid transfer in the intestine during hemorrhage is depicted in Fig 1 panel D A fluid gain to the blood stream occurred during the initial 25 min (maximal gain ≈ 0 5 ml/100 g tissue) after which a fluid loss occurred This loss averaged 6 ml/100 g of intestine after 90 min of hemorrhage —The initial passive decrease of regional blood volume (capacitance response) was 0 66 ± 0 05 ml/100 g tissue

Paw

Hemorrhage experiments were made on 8 cats In the control period arterial inflow pressure averaged 109 ± 5 mm Hg venous outflow pressure 5 ± 2 mm Hg blood flow 12 1 ± 3 6 ml/min 100 g and regional vascular resistance 8 7 ± 2 3 mm Hg/(ml/min × 100 g) Ar l and venous (great saphenous) plasma osmolality was 318 ± 2 and 321 ± 3 mOsm/kg H_2O respectively

In contrast to the resistance response in the intestine vascular resistance in the sym pathectomized α blocked paw increased markedly after hemorrhage and stabilized at a level about 500% above control after 15 min of hypotension (Fig 1 A) Regional blood flow thus decreased to very low values being about 0 8 ml/min × 100 g after 30 min This large increase of flow resistance was probably caused by local passive factors (*e g* elastic recoil cell aggregation etc) Evidence for such an interpretation was obtained from re gional hypotension experiments (see below) and further by the finding that blood flow quickly increased to the control level and roughly in parallel with the increase of the fusion gradient when the low perfusion pressure in hemorrhage was restored to n by transfusion

Plasma osmolality rose much more rapidly in the arterial than in the cutaneous blood which led to a clearcut positive arterio-venous osmolar gradient in the pa the first hour of bleeding (Fig 1 panel B) A net transcapillary fluid absorpti in the initial (< 40 min) period of hemorrhage (panel C) *i e* at the time when venous osmolar difference was most pronounced This suggests that the osmotic phenomenon an opinion further strengthened by the following similar increase of arterial plasma osmolality evoked by the administration glucose intravenously to non bled animals caused a fluid absorption of 0 0 100 g in the paw Late in hemorrhage a slight fluid filtration occurred (panel C)

tive fluid transfer in the paw is depicted in Fig 1 panel D At 40 min the total fluid gain from the paw to the circulation averaged 0 8 ±0 5 ml/100 g tissue it then slowly declined due to the transcapillary filtration loss and after about 80 min of hypotension the net fluid gain was zero The initial passive capacitance response was 1 1 ±0 14 ml/100 g in these experiments

Transcapillary fluid movements in the sympathectomized paw were also studied in non bled animals (n = 2) in response to regional arterial hypotension (50 mm Hg) maintained during 90 min This local hypotension caused no capillary fluid transfer during the first 5–10 min but later a net fluid filtration (0 02–0 06 ml/min × 100 g) Vascular resistance in the paw increased markedly also in these experiments e g by 200–400%

The figures for fluid transfer given above are calculated for 100 g soft paw tissue bone excluded

Discussion

This study has shown that a transcapillary fluid absorption is present in intestinal and cutaneous tissues in early stages of hemorrhage For the following reasons this absorption of extravascular fluid is believed to be caused at least mainly by an osmotic process evoked by the hemorrhagic arterial hyperosmolality (*a*) The regions were sympathectomized and the α adrenoceptors blocked which excluded fluid absorption by α adrenergic resetting of the pre /postcapillary resistance ratio (*cf* Öberg 1964) (*b*) The absorption took place in a period when the arterio-venous osmolar difference (reflecting the transcapillary osmolar gradient) was clearly positive (Fig 1 panels B and C) (*c*) When the arterial osmolality was increased in non bled cats to the same level as in hemorrhage by means of a slow intra venous infusion of hypertonic glucose a fluid absorption of a similar magnitude to that during bleeding was observed in the intestine and paw (*d*) Regional arterial hypotension (= 50 mm Hg) caused no fluid absorption in the paw nor does this occur in the small intestine as reported by others (Haglund and Lundgren 1972)

It may be argued that some non adrenergic vasoconstrictor substance (*e g* angiotension and/or vasopressin *cf* Bond Manley and Green 1967 McNeill *et al* 1970) could have caused a capillary pressure fall by an increase of the pre /postcapillary resistance ratio hence contributing to the fluid absorption Such an effect was apparently not present in the gut since hemorrhage led to an intestinal vascular resistance decline a finding in agreement with Haglund and Lundgren (1973) In the paw however a vascular resistance increase did occur but this seemed to be caused mainly by passive factors as a similar increase was evoked by regional hypotension *per se* the resistance increase in the latter case in fact was associated with a net fluid *filtration* indicating that in relative terms it was mainly confined to the postcapillary resistance vessels It cannot be entirely excluded that the intravascular instrumentation in the present experiments to some extent was responsible for the marked vascular resistance increase in the skin during hemorrhagic and regional hypotension If so such interference has led to some underestimation of the magnitude of the osmotic transcapillary fluid absorption in this tissue since the vascular resistance increase *per se* during hypotension caused a net fluid filtration thus opposing the osmotic absorption

Absorption of extravascular fluid was however, observed only during the first 20–40 min of hemorrhagic hypotension Later on fluid was lost from the blood stream into the extravascular space an effect particularly pronounced in the intestine (0 1 ml/min × 100 g tissue) This filtration most likely can be ascribed to an increased capillary hydrostatic pressure (P_c) both in the intestine and the paw An increase of P_c in the intestine thus is compatible with the observed decline of vascular resistance but mention should be made that this increase of P_c apparently amounted to less than 1 mm Hg. This can be deduced by taking into consideration the known large intestinal capillary filtration coefficient during bleeding (0 1–0 15 ml/min × 100g × mm Hg see Haglund and Lundgren 1973) The rise of P_c in the paw as mentioned may be due to passive factors (e g elastic recoil increased viscosity cell aggregation etc) which are known to preferentially affect the postcapillary resistance vessels (Eriksson and Lisander 1972)

In the present experiments the intestine and paw were sympathectomized and the a adrenoceptors blocked to facilitate the revelation of a possible osmotic absorption effect There are some earlier studies during hemorrhage on transcapillary fluid exchange in the intestine and paw with intact adrenergic innervation In the intact intestine most studies have revealed a net fluid filtration especially in late stages of hemorrhage (e g Johnson and Selkurt 1958 Glenert and Pedersen 1967 Cook Wilson and Taylor 1971 Haglund 1973) but some authors have reported a net fluid absorption (e g Johnson and Selkurt 1958 Marty and Zweifach 1971) During small bleedings of short duration (~10 min) Öberg (1964) found no effect whatsoever on the transcapillary fluid exchange a phenomenon which he attributed to a virtually unchanged pre to postcapillary resistance ratio Such bleedings however seems to cause small or negligable increases of plasma osmolality (Jarhult and Lundvall to be published) and hence no osmotic fluid absorption can be evoked In the intact cat paw (Öberg 1964) and dog foreleg (Schwinghamer et al 1970 Grega et al 1971) hemorrhage has been shown to cause a fluid absorption which was attributed to adrenergic resetting of the pre to postcapillary resistance ratio

Fluid transfer from the extravascular space of skeletal muscle into the blood stream is an important mechanism for plasma volume restoration in hemorrhage This effect is caused by a reflex adrenergic as well as a hyperglycemic osmotic mechanism each about equally important from a quantitative point of view (Järhult 1973) The present study suggests that hemorrhagic hyperglycemia causes an osmotic transcapillary fluid absorption also in the intestine and the skin Although this response thus is qualitatively similar in the 3 vascular beds the rate of transcapillary fluid absorption per unit tissue weight is higher in muscle than in skin and intestine and the effect is longer maintained in the former tissue during bleeding (Järhult 1973) These differences may at least partly be explained by the known dissimilarities in capillary ultrastructure The capillaries in skeletal muscle and skin are characterized by a continuous endothelium whereas they are fenestrated in the intestinal mucosa It seems likely that the reflection coefficient in fenestrated capillaries is lower than in such with continuous endothelium and that therefore the transcapillary osmotic fluid absorption is less pronounced It should be stressed that skeletal muscle is the most important tissue for the plasma volume restoration in bleeding simply due to its huge tissue mass. In fact it may be calculated that the transcapillary osmotic fluid withdrawal from all

intestine or all skin tissues would comprise less than 5 per cent of the concomitant osmotic fluid absorption from all skeletal muscles

The present study was supported by grants from the Swedish Medical Research Council (B75 14X —10-09A) and from the Faculty of Medicine University of Lund

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Acta physiol scand 1975 94 36–45

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Adrenergic and Cholinergic Innervation of the Rat Urinary Bladder

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Received 19 November 1974

Abstract

ALM P and M ELMÉR *Adrenergic and cholinergic innervation of the rat urinary bladder* Acta physiol scand 1975 94 36–45

The autonomic innervation of the rat urinary bladder was studied using histochemical methods and nerve stimulations A sparse adrenergic innervation of the detrusor muscle was found It was supposed to originate from long adrenergic neurones The trigonum area had a rich supply of adrenergic fibres probably derived from short adrenergic neurones. A uniformly rich supply of acetylcholine-esterase (AChE)-positive nerves was found in the whole bladder Postganglionic sympathetic denervation caused no detectable change of or AChE positive nerves in the bladder while parasympathetic decentralization or denervation duced a total disappearance of adrenergic fibres The AChE positive nerves were appreciably reduced in number after parasympathetic decentralization and not detectable after postganglionic denervation. Neither adrenergic nor AChE positive ganglion cells could be demonstrated in the bladder wall Electrical stimulation of the hypogastric nerves or the pelvic nerves distal to the pelvic ganglia elicited contraction of the detrusor muscle The responses were not affected by hexamethonium dihydroergotamine or propranolol but were slightly reduced by guanethidine reduced to about 40 by atropine and potentiated by eserine Stimulation of the pelvic nerve proximal to the pelvic ganglion was partially blocked by hexamethonium. It is concluded that the urinary bladder of the rat is supplied by postganglionic adrenergic fibres mainly via the pelvic nerves and only to a lesser extent via the hypogastric nerves Probably cholinergic fibres pass to the bladder mainly via the pelvic nerves but also via the hypogastric nerves having their cellbodies outside the bladder wall partly proximal to the pelvic ganglia

Key word Urinary bladder fluorescence histochemistry acetylcholine-esterase denervation nerve stimulation.

Physiological and pharmacological evidence indicates that the urinary bladder of the rat in contrast to other species is devoid of intramural ganglion cells (Vanov 1965 Carpenter and Rand 1965 Elmer 1973) These observations have been supported in morphological investigations by some authors (Carpenter and Rubin 1967 Chesher 1967) while others have found intramural cholinergic ganglion cells in the rat bladder (El Badawi and Schenk 1966)

Stimulation of the hypogastric nerve causes contraction of the bladder in the dog (Gianuzzi 1863) in the cat and monkey (Sherrington 1892) in the dog, cat and rabbit (Langley and Anderson 1895) and in the guinea pig (Mantegazza and Naimzada 1967) Vanov (1965) found no effect of hypogastric nerve stimulation in the rat In contrast to hypogastric nerve

stimulation injection of the sympathetic transmitter noradrenaline causes relaxation of the bladder in the dog (Dhasmana *et al* 1970) in the cat and the rabbit (Sigg and Sigg 1964 Edvardsen and Setekleiv 1968) and in the rat (Elmér 1974) Sigg and Sigg (1964) proposed that a cholinergic mechanism is involved in sympathetic nerve bladder transmission as postulated by Burn and Rand (1959) for other postganglionic sympathetic neuroeffector systems

In view of these results the present study was performed to get more information concerning the anatomical origin and distribution of adrenergic and acetylcholinesterase (AChE)-positive nerves of the rat urinary bladder using histochemical methods Furthermore the effects of electrical stimulation of the hypogastric and pelvic nerves on the urinary bladder before and after the administration of sympathetic and parasympathetic antagonists were studied

Methods

168 male albino rats of the Sprague Dawley strain weighing about 400 g were used. The histochemical investigations were performed in non-denervated bladders and in bladders denervated 10-14 days earlier To make a postganglionic sympathetic denervation the hypogastric nerves were cut distal to the hypogastric ganglia below the bifurcation of the aorta Parasympathetic decentralization was ach eved by the sectioning of the pelvic nerves proximal to the pelvic plexa, which in the male rat form distinct ganglia located on the lateral surface of the prostate gland (Langworthy 1965) In order to make a postganglionic denervation both pelvic ganglia we e extirpated (this procedure also interrupts the sympathetic fibres of the hypogastric nerves wh ch pass through the pelvic ganglia) The operations were performed aseptically in ether anesthesia. In the rats with divided parasympathetic nerves the bladder had to be emptied daily by manual pressure in ether anesthesia Despite this treatment these bladders always contained some residual urine and often weighed over 3 times more than controls when examined about 2 weeks after the nerves had been cut. To avoid bacterial infection the animals were given a sulfonamide (Sulfuno® Nordmark Werke 20 mg daily s c.)

The animals were killed by a blow in the neck whereupon the urinary bladders were rapidly dissected out and accord ng to Wakade and Kirpekar (197_) arbitrarily divided into neck, body and apical portion of the bladder Thereupon the tissue pieces were rapidly frozen in l quid isopentane cooled by liqu d nitrogen

For the histochemical demonstration of adrenergic nerves pieces of tissue were processed for the method of Falck and Hillarp (Falck *et al* 1962 Bjorklund *et al* 197) Serial sectioning was performed from each specimen In all the experimental groups 3 animals were gi en nialam de (Niamid® Pfizer Ltd 100 mg/kg i p 3 h before death) and L DOPA (L 3 4-dihydroxyphenylalanine S gma Chem Corp 40 mg/kg i v 1/2 h before death) in order to increase the intensity of fluorescence of the adrenerg c nerves (*J* M Imfors 1965)

Reserp ne (Serpasil® CIBA 0 05 mg/kg s c 16 h before death) and 6-hydroxydopamine (0 mg kg i v., 4 h before death) were tested as to their effects on the adrenergic nerve fluorescence in the bladder compared to heart atria, submaxillary gland pancreas, vas defer ns and sem nal ves cle All tissue pieces wer processed for fluorescence histochem stry as described abo e

In three rats pieces of t sue were taken from the hypogastric nerves and the pel ic nerves proximally and d stally to the places whe e these nerves w re cut in the denervation operations. The specimens obtained were processed fo the method of Falck and H llarp To avo d a reduct on in the fluorescence ntensity of the muscular adrene gic bladder nerves (see below) the mod f'cations of Bjorklund and Falck (1968) were used n the paraffin embedd ng step and when mounting the sect ons

For the demon tration of acetylchol ne-este ase (AChE) act vity the copper th ochol ne method of Koelle and Friedenwald (1949) as modif ed by Holmstedt (1957) wa used Cryostate sect ons (10 μm) were incubated fo I 16 h in acetylthiochol ne in the presence of M pafox® (bis-mo o sopropylam de phosphoryl fluoride) for the inhib tion of nonspec fic chol nesterase (Holmstedt 1957 Holmstedt and Sjoqu st 1961) or BW 84 C 51 (1,5-b 4-allyldimethylammo umphenyl pentan 3-on d prom de Wellcome Res Lab Beckenh m England) a spec fic inhib tor of AChE (Bayliss and Todrick 1956) Eosin was used as counterst n mounting was performed in Entellan® (Merck)

For the stimulation experiments 58 rats were anesthetized with chloralose (100 mg/kg) given through a cannula in a femoral vein after induction with ether. The bladder was exposed and the ureters were ligated. A glass cannula was inserted into the bladder through an incision in the urethra. The bladder was filled with 0.5 ml of physiological saline and the pressure developed by the detrusor muscle was recorded by means of a transducer and a polygraph. The resting pressure was about 10 mm Hg in all experiments.

The hypogastric nerves were cut distal to the hypogastric ganglia below the bifurcation of the aorta and the distal ends were stimulated electrically using a bipolar electrode. The left pelvic nerve was stimulated after section proximal or distal to the pelvic ganglion. In order to avoid stimulating the sympathetic fibres from the hypogastric nerves which pass through the pelvic ganglia, the hypogastric nerves were cut 10 days before the pelvic nerve stimulations allowing the sympathetic fibres to degenerate. A Grass stimulator giving rectangular pulses with a duration of 2 ms, a frequency of 0.1–20 Hz and of supramaximal voltage (5–10 V) was used.

For statistical evaluation of the data Student's t test for paired samples was used. The 0.05 level of probability was accepted as significant.

Drugs. The substances used in the stimulation experiments were: eserine sulphate, hexamethonium bromide, dihydroergotamine methansulphonate, phenoxybenzamine hydrochloride, propranolol hydrochloride, guanethidine bisulphate, atropine sulphate, Hoechst 9980 (piperidino-ethyl-diphenyl a etamide) and prilocain chloride (Citanest®, Astra, Sweden). The drugs were injected through the cannula in the femoral vein except prilocain which was applied locally on the nerve distal to the site of stimulation.

Results

Histochemical findings

Adrenergic nerves. In non-denervated animals fine varicose nerve terminals very sparsely occurred in the smooth muscular layers throughout the whole bladder except the trigonum area (see below), mostly running between the bundles of smooth muscle cells in their longitudinal direction, sometimes running across the muscle cells connecting longitudinally running terminals, thus forming a very slender plexus around the muscular fibres (Fig. 1 A). However, in the trigonum area the supply of adrenergic nerve terminals of the smooth muscular layers was very rich, clearly exceeding that of other parts of the bladder. Furthermore, in the trigonum area the varicosities of the adrenergic nerve terminals were clearly more coarse than in the other bladder parts. Fine varicose adrenergic nerves also appeared around some of the blood vessels in the submucosa, muscular and adventitia layers. Despite serial sectioning, no adrenergic ganglion cells could be demonstrated within the rat bladder wall. On the other hand, clusters of ganglion cells could frequently be found outside the bladder wall in the connective tissue surrounding the base of the bladder. In the muscular layers clusters of small intensely fluorescent cells (SIF-cells) could sometimes be found. Treatment with nialamide and L-DOPA did not appear to increase the number of adrenergic nerves. In the specimens from the hypogastric and pelvic nerves bundles of nerves with a specific greenish fluorescence were found.

After pretreatment with reserpine or 6-hydroxydopamine no adrenergic nerve fluorescence was seen in the apical and body portion of the bladder, nor could any fluorescence be found in heart atria, submaxillary gland and pancreas. However, in the trigonum area of the neck portion of the bladder and in the seminal vesicle and vas deferens the dense adrenergic innervation of the smooth muscular layers was appreciably unchanged. Moreover, some of the blood vessels (smaller arteries and arterioles) in the submucosa of the trigonum area displayed no adrenergic nerve fluorescence.

Postganglionic sympathetic denervation did not appear to influence the supply of either





Fig. 1 A Muscular layers of rat bladder wall. Sparse distribution of adrenergic nerve terminals compared to AChE positive nerves (see Fig. 1 B) Fluorescence micrograph × 140

Fig 1 B Section through rat bladder wall Rich distribution of AChE positive nerves are seen except in the most apical part which consists of the mucosa layer The large dark spots are AChE positive nerve trunks Light micrograph × 88

vascular or muscular adrenergic nerves in any bladder region whether the animals were pretreated with nialamide and L-DOPA or not After parasympathetic decentralization or postganglionic denervation no adrenergic nerves were detected even if the animals were pretreated with nialamide and L-DOPA

AChE positive nerves The number of nerves demonstrating AChE activity very distinctly exceeded that of nerves demonstrating adrenergic fluorescence (Fig 1 B) Along the bundles of smooth muscle cells in the muscular layers AChE positive nerve fibres regularly appeared originating from larger trunks of AChE positive nerve fibres lying most basally in the muscular layers No apparent differences as to the number and distribution of AChE positive nerves could be found between the different regional parts of the bladder In addition in some of the vessels there could be observed small AChE positive branches ending in the adventitia No AChE positive ganglion cells could be demonstrated within the bladder wall despite serial sectioning

Postganglionic sympathetic denervation did not seemingly influence the supply of AChE positive nerve fibres Parasympathetic decentralization produced an appreciable reduction in the number of AChE positive nerve fibres However ... AChE ...

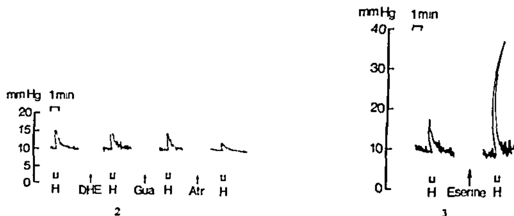


Fig 2 Pressure responses of the rat urinary bladder to electrical stimulation of the hypogastric nerves (H) at 15 Hz. DHE dihydroergotamine 2 mg/kg Gua guanethidine 1 mg kg Atr atropine 1 mg kg

Fig 3 Pressure responses of the rat urinary bladder to electrical stimulation of the hypogastric nerves (H) at 15 Hz before and after the injection of eserine 0 2 mg/kg

nerve fibres could be seen the number apparently somewhat greater in the neck than in the other bladder portions After postganglionic denervation there was an almost complete disappearance of AChE positive nerve fibres

Stimulation experiments

Sympathetic stimulation Electrical stimulation of the hypogastric nerves caused contraction of the detrusor muscle Maximal pressure response was obtained using a stimulation frequency of 15 Hz, which gave an increase in the bladder pressure of 5 6 ± 0 7 mm Hg (mean ± S E n 27)

The responses to stimulation of the hypogastric nerves were not affected by previous injection of hexamethonium 10 mg/kg, dihydroergotamine 2 mg/kg, phenoxybenzamine 5 mg/kg or propranolol 2 mg/kg As shown in the table guanethidine 1 mg/kg reduced the response by about 25 %. Atropine in a dose of 0 1 mg/kg reduced the response by about 40 % and after the injection of 1 mg/kg the reduction was about 60 %. The parasympatholytic agent Hoechst 9980 (Schaumann and Lindner 1951) had the same effect The pressure responses to electrical stimulation of the hypogastric nerves before and after the injection of dihydroergotamine guanethidine and atropine are shown in Fig 2

After injection of 0 2-0 4 mg/kg of eserine the response to stimulation of the hypogastric nerves at 15 Hz was increased about 3 times (Fig 3)

Parasympathetic stimulation Electrical stimulation of one of the pelvic nerves proximal or distal to the pelvic ganglion caused contraction of the detrusor muscle Maximal pressure response was obtained with a stimulation frequency of 15 Hz increasing the intravesical pressure by 36 4 ± 2.5 mm Hg (n = 28)

The responses to stimulation of the pelvic nerve distal to the pelvic ganglion were not affected by previous injection of hexamethonium 10 mg kg dihydroergotamine 2 mg kg or propranolol 4 mg kg. Guanethidine 1 mg kg caused a reduction of the response of

TABLE I Pressure responses in mm Hg in the rat urinary bladder to electrical stimulation at 15 Hz of the hypogastric or pelvic nerves before and after the injection of guanethidine 1 mg/kg, or atropine 1 mg/kg. Values are mean ± S E n = number of rats p = significance

	Guanethidine		n	p	Atropine		n	p
	Before	After			Before	After		
Hypogastric n	1 6 ± 0 4	1 . ± 0 3	7	< 0 05	3 8 ± 0 6	1 5 ± 0 3	8	< 0 01
Pelvic n	26 4 ± 3 8	24.2 ± 3 7	12	< 0 001	-9 6 ± 3 9	12 0 ± 1.5	13	< 0 001

about 8 Atropine reduced the response by about 60% in a dose of 0 1 mg/kg and the injection of 1 mg/kg did not cause any further reduction (Table I) When both guanethidine and atropine were given to the same rat the effect of each drug was the same independently of which drug was given first When the pelvic nerve was stimulated proximal to the pelvic ganglion hexamethonium reduced the response by 50–75% and the reduction was bigger the more proximal to the ganglion the nerve was stimulated

Eserine increased the response to stimulation of the pelvic nerve distal to the pelvic ganglion by about 50 % in the doses indicated above

After application of the local anesthetic agent prilocain on the hypogastric or pelvic nerves no response to electrical stimulation of the nerves was obtained, while direct stimulation on the bladder wall still caused a contraction

Discussion

In agreement with earlier findings the smooth muscular layers of the rat urinary bladder has a very sparse supply of adrenergic nerves except in the *trigonum area* where it is much richer (*cf* Hamberger and Norberg 1965 a El Badawi and Schenk 1966 Owman and Sjoberg 1972, Wakade and Kirpekar 1972, Sundin and Dahlstrom 1973) Furthermore there is a rich supply of AChE positive nerves in all parts of the bladder very clearly exceeding that of adrenergic nerves (*cf* El Badawi and Schenk 1966)

The finding that the responses to electrical stimulation of the hypogastric and pelvic nerves were partially blocked by guanethidine as well as by atropine might also suggest the presence of both adrenergic and cholinergic fibres A reduction of the response to pelvic nerve stimulation after atropine in the rat has been described earlier (Vanov 1965) An atropine sensitive portion of the response to transmural stimulation of the urinary bladder *in vitro* was found by Chesher (1970) in the rat but not in the cat and guinea pig

Parasympathetic decentralization and total postganglionic denervation produced a complete disappearance of adrenergic nerves but seemingly no effect was achieved at post ganglionic sympathetic denervation This probably means that the rat detrusor muscle is mainly supplied by adrenergic nerves *via* sacral nerve trunks and not *via* the hypogastric nerves This could be in line with the findings in the present work demonstrating bundles of adrenergic nerve fibres running in the pelvic nerves It is also in accordance with previous findings in cats (Hamberger and Norberg 1965 b Sundin and Dahlstrom 1973) but is

trary to findings in guinea pigs (Wakade and Kirpekar 1972) Furthermore the total disappearance of adrenergic nerves after parasympathetic decentralization and total postganglionic denervation in connexion with the seeming absence of adrenergic ganglion cells within the bladder wall suggest that the adrenergic nerves of the rat bladder wall are peripheral postganglionic fibres (*cf* El Badawi and Schenk 1966)

Because of anatomical and functional differences the neurones of the peripheral adrenergic nerve system can be divided into long and short adrenergic neurones (see review by Owman *et al* 1974) Long adrenergic neurones have a short preganglionic and a long postganglionic fibre Furthermore the ganglion cells are localized far away from the organ to be innervated In the short adrenergic neurones the situation is the reverse In view of this the adrenergic nerve terminals in heart submaxillary gland and pancreas are supposed to originate from long adrenergic neurones and the adrenergic nerve terminals to seminal vesicle and vas deferens are supposed to originate from short adrenergic neurones (Sjostrand 1965 Owman *et al* 1974) In small doses reserpine and 6-hydroxydopamine are known to deplete the transmitter fluorescence of nerve terminals of long adrenergic neurones but not (or to a much slower velocity) in nerve terminals of short adrenergic neurones (Owman and Sjoberg 1967 Malmfors and Sachs 1968 Sjostrand and Swedin 1968 Jane *et al* 1970 Jonsson and Sachs 1970) Consequently from the present findings it can be supposed that the adrenergic innervation of the muscular layers and of some of the blood vessels in the trigonum area originates from short adrenergic neurones as no appreciable effect on the adrenergic nerve fluorescence could be demonstrated by reserpine or 6-hydroxydopamine and nor could any effects of these drugs be found in the seminal vesicle or vas deferens On the other hand fine varicose adrenergic nerve terminals of the smooth muscular layers in the other parts might originate from long adrenergic neurones as no adrenergic nerve fluorescence was simultaneously seen in these bladder parts (nor in the heart atria submaxillary gland or pancreas) after treatment with reserpine or 6-hydroxydopamine

Despite serial sectioning only SIF-cells and no conventional adrenergic ganglion cells were found intramurally in the bladder wall This verifies earlier studies on the rat bladder wall (El Badawi and Schenk 1966) Although SIF-cells have been found in a lot of various organs their physiological role is still unknown

The response to stimulation of the hypogastric nerves was a contraction of the detrusor muscle in the rat as is most often the case in other species (Giannuzzi 1863 Sherrington 1892, Langley and Anderson 1895 Mantegazza and Naimzada 19(7) Vanov (1965) did not obtain any effect of hypogastric nerve stimulation when recording the responses of the rat bladder as contractions in the axis from the urethra to the vertex instead of changes in the intravesical pressure Some authors have found a relaxation of the bladder after an initial contraction in the dog and cat (Griffiths 1895 Kuntz and Saccomanno 1944) The response in the present study was not affected by the ganglionic blocking agent hexamethonium indicating that the fibres stimulated were postganglionic Since the urinary bladder of the rat contains both excitatory α adrenoceptors and inhibitory β-receptors (Elmér 1974) an effect of adrenergic blocking agents on the response to hypogastric nerve stimulation could be expected Neither the α receptor blocking agents dihydroergotamine or phenoxybenzamine nor the β-receptor blocking agent propranolol however had any significant effect

on the detrusor contraction caused by hypogastric nerve stimulation Furthermore the adrenergic neurone blocking drug guanethidine caused only a slight reduction of the response while atropine and the specific parasympatholytic agent Hoechst 9980 (Emmelin and Stromblad 1957) markedly reduced it This is in agreement with the histochemical findings suggesting that the main part of the adrenergic nerves to the urinary bladder runs *via* the pelvic nerves The slight reduction of the response to pelvic nerve stimulation caused by guanethidine also might support this concept although no effect of adrenoceptor blocking agents was found The dose of guanethidine used was probably sufficiently low not to have any local anesthetic effect (Bein 1960) The existence of sympathetic fibres outside the hypogastric nerves was predicted by Giannuzzi (1863) on the basis of stimulation experiments in the dog

The AChE positive nerves were clearly reduced at parasympathetic decentralization almost totally absent at total postganglionic denervation but apparently not changed in number at postganglionic sympathetic denervation This probably means that most of the AChE positive nerves pass to the bladder *via* sacral nerve trunks and that a great part of them have their cellbodies located distant from the pelvic plexa Furthermore it also suggests that the AChE positive nerves in the bladder wall are postganglionic fibres (*cf* El Badawi and Schenk 1966)

The bladder response to both hypogastric and pelvic nerve stimulation was increased by eserine suggesting that cholinergic fibres were stimulated in both nerves It is not likely that the potentiation of the response occurred at a ganglionic level since both the histochemical findings and the hexamethonium experiments indicated that the nerves stimulated in this case were postganglionic When the pelvic nerve was stimulated proximal to the pelvic ganglion the response was not totally abolished by hexamethonium, indicating that some of the axons in the pelvic nerve originate from ganglion cells proximal to the pelvic ganglion in agreement with the histochemical findings

Since the atropine resistance at hypogastric nerve stimulation was not greater than that of the pelvic nerve it is still possible that the nonadrenergic portion of the hypogastric nerve may be cholinergic Even if this is the case the number of cholinergic nerve fibres in the bladder wall originating from the hypogastric nerves might be expected to be much smaller than that from the pelvic nerves and this may explain why no reduction of AChE positive nerves could be detected at sympathetic denervation

Evidence of a non adrenergic mechanism involved in sympathetic nerve bladder transmission has been described in the cat (Sigg and Sigg 1964 De Sy 1972, Sundin and Dahlstrom 1973) and guinea pig (Mantegazza and Naimzada 1967) In these species the bladder wall contains intramural ganglia and atropine reduced the response only at such high doses that a ganglionic blocking effect can be expected (Feldberg and Vartiainen 1935) In the cat the hypogastric response is blocked by guanethidine and bretylium but not by the α blocking agent phentolamine (De Sy 1972) An adrenergic stimulating action on cholinergic ganglion cells in the bladder wall was suggested, since adrenergic nerve terminals forming synaptic structures around non adrenergic ganglion cells have been demonstrated histochemically in the cat (Hamberger and Norberg 1965 a) In the rat bladder however where no evidence of intramural ganglia were found the non adrenergic part of the postganglionic sym

nerve bladder transmission might be explained by the presence of postganglionic cholinergic fibres in the hypogastric nerves Hypothetically adrenergic nerve fibres may influence cholinergic nerve activity peripherally as described in the heart (Leaders 1963) the influence being inaccessible to adrenoceptor blocking agents

The part of the response to hypogastric and pelvic nerve stimulation that could not be blocked by any of the sympathetic or parasympathetic antagonists used was totally abolished by the local anesthetic agent prilocain indicating that the bladder stimulation was nerve mediated and not due to any direct effect on the detrusor muscle

This work was supported by grants from the Medical Faculty University of Lund

For generous supply of drugs the authors thank Nordmark Werke Hamburg (Sulfuno®) Swedish CIBA (Serpasil®) and AB Hässle (6-hydroxydopamine)

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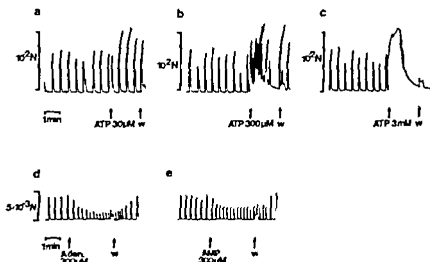


Fig. 1 a) b) and c) The effect of ATP on spontaneous mechanical activity in normal PSS A low a medium and a high concentration is shown d) and e) The effect of adenosine and AMP 300 µM on spontaneous activity in normal PSS Each substance is added at the first arrow and washed away at the second Time and tension scales are shown by the bracketed bars.

expressed as the increase in mean tension in percent of maximal tension development induced by 10 µM noradrenaline (NA) ATP was about 2 2 times more potent than ADP At medium concentrations ADP sometimes lacked the initial contraction and only an increase in the quency of contractions resembling the effect of AMP was seen

The effect of ATP 300 or 1 000 µM was also tested for periods up to 15 min It was found that after the initial excitation a short period of inhibition of mechanical activity followed After 4 5 min however activity started anew with small contractions of high frequency resembling the effect of AMP (see below)

The effect of AMP and adenosine on spontaneous activity in normal solution

The effects of AMP and adenosine were qualitatively the same but opposite those of ATP and ADP Both substances in concentrations from 10 µM and upwards decreased the amplitude of the spontaneous contractions but increased their frequency (Fig 1 d and e) The effects were graded according to dose and 300 µM caused a 50% reduction in the contraction amplitude while the frequency of contractions was roughly doubled Further increase in the drug concentration did not increase the response (see Fig 2 b and Table I) The portal vein showed tachyphylaxis towards both AMP and adenosine when the interval between stimulations was less than 7 8 min or when the organ bath was only rinsed once following a medium or maximal concentration In contrast to ATP and ADP there was also a slight decline in the response to AMP and adenosine during the course of the experiment The inhibition caused by AMP or adenosine did not however change during the stimulation period even when the drug was present for periods up to 15 min The effects of 5 different concentrations of AMP and adenosine on contraction amplitude are summarized in Fig 2 b while Table I summarizes their effects on contraction frequency

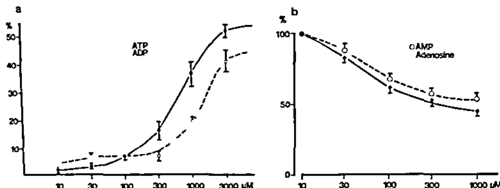


Fig 2 a) The dose response curves for ATP (filled circles) and ADP (open circles) Abscissa Concentration in μM Ordinate Average tension during first min of response in percent of maximal tension response to 10 μM NA b) The effect of adenosine (filled circles) and AMP (open circles) on contraction amplitude in normal PSS Abscissa Concentration in μM Ordinate Contraction amplitude in percent of control In both figures each point represents the mean of 8 muscles given with ± S E.

The effects of ATP ADP AMP and adenosine on the K contracture

When K high solution (128 mM K^+) is applied to the rat portal vein the muscle depolarizes and a smooth contracture develops After a transient phasic peak contraction the contracture stabilizes at about 40 % of the initial maximal tension (Axelsson *et al* 1967) During the stable part of the contracture ATP ADP AMP and adenosine were applied at a concentration of 300 μM The contracture was allowed to stabilize after each stimulation ATP and ADP increased the contracture while AMP and adenosine relaxed it The increase in tension was for ATP 19 1 ±4 4 (4) and for ADP 12 0 ±1 6 % (4) of baseline tension, while AMP relaxed the contracture by 8 7 ±2 7 % (4) and adenosine by 8 4 ±0 6 % (4)

The effects of ATP AMP and adenosine in solutions with varying Mg and Ca^{++} concentrations

Reduction or removal of Mg^{++} from the normal PSS increases the amplitude and duration of the spontaneous contractions while the frequency is usually unchanged When ATP 300 μM was added in these conditions the drug effect was potentiated Upon return to normal PSS the response to ATP decreased to control On the other hand when the Mg^{++} concentration was raised to 4 8 mM *i e* 4 times normal the spontaneous activity was drastically reduced and in a few cases completely inhibited ATP in the same concentration as before had in this solution a very small effect

A whole series of experiments were performed in which the Mg and Ca concentrations of the external medium were systematically varied ATP 300 μM was added for 3 min in each solution after the spontaneous activity had stabilized and the response to ATP after subtraction of the spontaneous activity in the corresponding solution compared to the maximal tension response to NA in the respective solution The results are summarized in Fig 3 From these results it is evident that the ATP effect is inversely related to the external Mg concentration The ATP effect however was reduced in Ca high as well as in C

TABLE I The effect of AMP and adenosine on the frequency of contraction All values expressed as the mean percentage increase in frequency caused by the drug ± 2 S E (n = 5)

μM	10	30	100	300	1 000
AMP	—	28 ± 6	69 ± 13	124 ± 6	90 ± 18
Adenosine	8 ± 5	41 ± 8	101 ± 25	124 ± 14	94 ± 17

PSS as compared to normal solution Similar experiments were performed with adenosine and AMP but there were no changes in the effects of those substances neither with $(Mg^{++})_o$ nor with $(Ca^{++})_o$

The effects of ATP in varying Mg^{++} concentrations were also studied on the K-contracture Mg free or Mg high solutions *per se* did not shift the baseline of the contracture When ATP 300 μM was added the effect was in all solutions a transient contraction of constant magnitude Thus in the depolarized tissue external Mg^{++} had no influence on the ATP effect

Influence of ATP and adenosine on the response to exogenous noradrenaline and acetylcholine

Acetylcholine (ACh) ACh has an excitatory effect on the rat portal vein similar to the effect of NA causing a large summated contraction To test if there was any interaction between ACh and ATP or adenosine these substances were added 2 min prior to 2 μM ACh and the response compared to a control ACh application It was found that the effect of ATP up to 1 mM was simply additive to that of ACh while adenosine partly inhibited the ACh contraction The inhibition was 26 ± 6% (5) for 300 μM adenosine and 42 ± 10 (4) for 1 mM adenosine

Noradrenaline (NA) Similar experiments as with ACh were performed with 1 μM NA It was found that the effect of ATP was additive to that of NA On the other hand adenosine or AMP did not as in the case of ACh cause partial inhibition On the contrary the effect on NA was in *some* preparations potentiated while the normal response was an unchanged contraction in the adenosine pretreated muscle The interaction between 1 μM NA and 300 μM adenosine was (also) tested in 5 0 mM Ca and 0 5 mM Ca PSS It was found that in Ca high solution the response to NA was significantly potentiated the ratio between adenosine treated and control stimulations being 1 15 ± 0 04 (7) In Ca low solution adenosine had no effect on the response to NA

Interaction of ATP ADP AMP and adenosine with adrenergic and cholinergic blockers

The effects of ATP ADP AMP and adenosine 300 μM were studied in the presence and absence of phenoxybenzamine (adrenergic alfa blocker) propranolol (adrenergic beta blocker) or atropine (cholinergic muscarinic blocker) The blockers were added at a concentration of 10 μM which completely blocked all responses to added NA or ACh The blockers had no effect either single or in combination on the response to ATP ADP AMP or adenosine

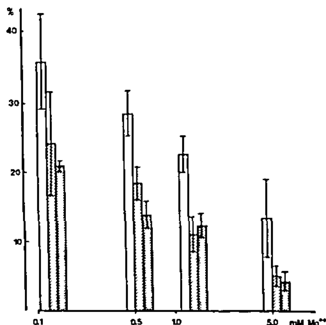


Fig 3 The effect of Mg and Ca on the excitatory response to ATP 300 μM in normal PSS (empty columns) Ca 0 5 mM (striped columns) and Ca 5 mM (hatched columns) The vertical bars represent ± S E Abscissa Mg^{++} concentration in mM Ordinate A erage tension during first min of ATP response in percent of maximal contraction

Effects of theophylline

Theophylline is known to be a potent phosphodiesterase inhibitor causing accumulation of 3 5 cyclic AMP in the cell When theophylline 100 μM was added and the response to ATP and adenosine 300 μM after theophylline compared to control responses it was found that theophylline significantly potentiated the frequency increase caused by adenosine by 14 ±4 (6) and the decrease in amplitude by 36±3% (6) Theophylline *per se* slightly reduced spontaneous activity and did also decrease the response to ATP by about one third

The effects of related compounds on spontaneous activity and K contracture

To see if the shown effects were specific for the substances used GTP GMP guanosine guanine 3 5 -cyclic AMP and adenine were tested on the spontaneous activity and on the K-contracture in 4 preparations The concentration for all substances was 300 μM GTP guanine and 3 5 -cyclic AMP had virtually no effect neither in the polarized nor in the depolarized preparation GMP guanosine and adenine had a slight relaxing effect on the contracture and an AMP like effect on spontaneous activity Guanosine 300 μM was tested on spontaneous activity and compared to adenosine in the same concentration Adenosine was found to be 5 0 times more potent in increasing contraction frequency and 2 7 times more potent in reducing contraction amplitude

Discussion

The results obtained in this study show that there is a difference between the effects of ATP and ADP on one hand and AMP and adenosine on the other ATP and ADP alway

an initial excitation while AMP and adenosine inhibited spontaneous activity The direction of the response for the two groups of substances was independent of the ionic milieu and membrane polarization

It is possible that the contraction induced by ATP in the rat portal vein is due to a direct effect on Ca + influx or Ca+ release Other possibilities should however first be evaluated Thus ATP might interfere with the release of noradrenaline (or acetylcholine) from nerve endings but since none of the blockers tried affected the ATP response this is unlikely The effects of ATP were also simply additive those of NA and ACh at the concentrations used above

The question whether the effect of ATP could be due to energy liberation from the phosphate bonds was not posed in this study However readmission of glucose after a period of glucose depletion inhibits spontaneous activity (Wahlström unpublished) and it is therefore not likely that energy liberation from ATP would cause an excitatory response

ATP may bind Mg in or at the membrane as proposed by Daniel and Irwin (1965) The results obtained in this study confirm this idea In the rat portal vein the ATP induced contraction was obviously potentiated by decrease or removal of external Mg We also found however that the effect of ATP decreased in Ca high solution irrespective of the Mg -concentration This result might be explained by assuming that part of the ATP added is chelated by Ca at this high Ca -concentration but it is more likely to be due to an increased membrane stability caused by increased Ca binding at permeability-controlling sites in the high Ca solution as proposed for guinea pig taenia coli by Bülbring Tomita (1969)

The fact that ATP increases the K -contracture the magnitude of the response being independent of the Mg -concentration could suggest an intracellular action of ATP However as shown by Durbin and Jenkinson (1961) drugs—in their case Carbachol—can increase the Ca uptake in smooth muscle and thus augment the tension by a membrane mechanism even in the depolarized condition

AMP and adenosine had inhibitory effects on the rat portal vein except that adenosine potentiated the NA induced contraction on 5 0 mM Ca solution The latter results is similar to that obtained in the guinea pig vas deferens by Iso (1973) but the explanation for this phenomenon has yet to be found Since AMP and adenosine hardly penetrate the cell membrane (Roll *et al* 1956) the inhibitory effects in normal solution are probably exerted at the membrane and linked with the adenosine moiety This might also explain why 3 5 cyclic AMP had no effect in normal PSS because this compound is structurally different from AMP and adenosine

The potentiation by theophylline of the AMP and adenosine effects indicates that an intracellular action is also possible AMP and adenosine might through a membrane mechanism stimulate adenyl cyclase This will increase Ca binding to intracellular structures thereby decreasing the intracellular Ca -concentration (Andersson 1972) A similar mechanism decrease in [Ca] was proposed to explain the effects of isoprenaline on the rat portal vein on the basis of the similarity between the effects of isoprenaline and low [Ca] (Johansson *et al* 1967) The records shown in fig 1 d and e above also strongly resemble the effects of a moderate decrease in [Ca] The AMP and adenosine effects showed however

no correlation with external [Ca+]o and the evidence for such a mechanism therefore is only tentative

This work was supported by grants from the Swedish Natural Sciences Research Council (∠447–010) from Lars Hi rtas Minne and from Wilhelm och Martina Lundgrens Vetenskapsfond The excellent technical assistance of Mrs Gunilla Rydgren is gratefully acknowledged

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Acta physiol scand 1975 94 54-62

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Incorporation of Choline, Serine, Ethanolamine and Inositol into Phospholipids of Isolated Rat Mast Cells

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Received 6 December 1974

Abstract

STRANDBERG KJ A SYDBOM and B UVNAS *Incorporation of choline serine ethanolamine and inositol into phospholipids of isolated rat mast cells* Acta physiol scand 1975 94 54-62

The incorporation of labelled phospholipid precursors, [Me ^{14}C]-choline L [3-^{14}C] serine [[illegible] ^{14}C]-ethanolamine and [[illegible] ^{3}H]-myoinositol into the phospholipids of isolated rat mast cells was studied. The label from the different precursors were found to be essentially associated with compounds with the [illegible] of the respective phospholipids. Whereas the incorporation of [Me ^{14}C]-choline and L [3 ^{14}C] serine showed evidence of saturation the incorporation of [[illegible] C]-ethanolamine was linear with time ([illegible] h) and it was not saturated by increasing the concentration from 0.07 mM to [illegible] 07 mM. The incorporation of [[illegible] ^{3}H] myoinositol was stimulated by Ca^{2+} (1 mM) or Mg^{2+} (1 mM), while the incorporation of the other precursors was stimulated only in the presence of both Ca^{2+} and Mg^{2+} (1 mM). Antimycin A (1 μM), an inhibitor of the respiratory chain significantly inhibited the incorporation of [Me ^{14}C]-choline L [3-^{14}C] serine and [[illegible] H] myoinositol but not that of [[illegible] ^{14}C]-ethanolamine. The experimental system used might be a useful model for studies on the turnover of membrane phospholipids during histamine release

Histamine release from rat mast cells can be induced by a variety of agents. Due to the morphological effects produced and or the biochemical conditions required they have been classified as specific and non specific releasers. Antigen and compound 48/80 are dependent on energy requiring reactions in the mast cell for their action. The precise nature of the release mechanism triggered by these agents remains to be ascertained. The participation of membrane bound enzymes *i e* an esterase and a phospholipase A like enzyme and contractile elements in the process has however been implied (for references see Becker and Henson 1973)

The appearance of biologically active lipid soluble principles on histamine release induced by antigen or compound 48/80 has been suggested to indicate the activation of a hydrolytic enzyme during the release (Uvnas and Thon 1959 Änggård *et al* 1963). Part of the active principles consists of prostaglandins (Piper and Vane 1969 Änggård and Strandberg 1971) compounds known to be synthetized from fatty acids split off from membrane phospholipids by a hydrolytic enzyme (Lands and Samuelsson 1968). If a phospholipase

were activated in the mast cell during the histamine release changes in the phospholipid composition of the cells might be anticipated In the present experiments the incorporation of labelled phospholipid precursors into rat mast cell phospholipids has been investigated in order to obtain a suitable experimental system for recording possible changes in the phospholipid pattern on histamine release

Materials and Methods

[Me ^{14}C]-choline chloride (61 mCi m mol) [^{14}C]-ethanolamine hydrochloride (30 mCi m mol) L [3- ^{14}C] serine (48 mCi m mol) and [2 ^{3}H]-myo nositol (4 5 Ci m mol) were purchased from the Radiochemical Centre Amersham England The radiochemical purity of the substances were tested by paper chromatography in different solvent systems. ^{14}C-choline (99% purity) n butanol acetic acid water-ethanol 80 10 30 0 (v/v) ^{14}C-ethanolamine (99%) and ^{14}C serine (99%) n butanol acetic acid water 20 30 50 (v/v) H inositol (92) n butanol-ethanol water 104 66 30 (v/v)

Phosphat dylinositol. General Biochemicals Chagrin Falls Ohio USA. Phosphatidylethanolamine and phosphatidyl L serine Koch Light Lab Ltd. England. L α Lecithin (synthetic) lysolecithin lysophosphatidylethanolamine and sphingomyelin Sigma Chem. Comp Inc MO USA

Insta Gel, Packard Instrument Comp Inc Ill. USA The scintillator solution toluene PPO-POPOP consisted of 0 4 2,5-diphenyloxazole (PPO) and 0 01% 1 4-di-(2-(4-methyl 5 phenyloxazolyl))-benzene (POPOP) in toluene PPO and POPOP were obtained from Koch Light Lab Ltd England

Ant mycin A was obtained from Nutrit onal B ochemical Corp., Cleveland USA It was dissolved in ethanol (96% v/v) The final concentration of ethanol in the incubation medium was 0 1 both in antimycin A and control samples

Ficoll was purchased from AB Pharmacia, Uppsala, Sweden and human serum albumin (free from preservatives) from AB Kab Sweden. Sol ents were Merck p a quality and were used without purif cation

I olat on of mast cells

Mast cells from the peritoneal and pleural cavities of male Sprague Dawley rats (350–400 g) were isolated as described by Thon and Uvnäs (1967) After gradient centrifugation on Ficoll (350 g) the cells were washed twice at 20 C with a 9/1 (v v) mixture of isotonic saline containing 1 mg of human serum albumin and 67 mM Sörensens phosphate buffer pH 6.3

I bation p oced e

An aliquot of the mast cell suspens on contain ng about 2 mill on cells in 0 5 ml was incubated with 2 μ Ci of either C-choline C ethanolam ne or ^{14}C serine or with 10 μ Ci of ^{3}H inositol in a water bath (1 0 reversals min) at 37 C for 30 min unless otherwise stated

The standard incubation medium was a salt solut on (NaCl 137 mM KCl 2.7 mM $CaCl_2$ 1 8 mM $MgCl_2$ 1 mM) contain ng 10% (/) Sorensens phosphate buffer (67 mM) pH 7 0 human serum al umin (1 mg/ml) and glucose (1 mg ml) The i cubation was terminated by placing the tubes in ice water and add ng 1 5 ml of the ice-cold med um.

Ext act on of pho ph l pids

After the incubation the cells were centrifuged (350 × g) at 4 C for 10 min The supernatant was d scarded and the cells were washed twice with 2 ml of the ice-cold medium. The rad oactiv ty in the washings was counted to establ sh that non incorporated material was l minated After wash ng the wa ls of the tubes were thoroughly dried by the use of strips of filter paper One ml of isotonic N Cl was added and the cells were counted in a Burker chamber 0 .5 ml of d stilled water was added to the cell suspensio and the cells were son cated at 4 C using a MSE ultrasonicator (8 μ m for 10 s) Aliquots (0 1–5 ml) of the suspension were taken for determ nat on of radio ct ity and prot n (Lowry t al 1951) The rem ining suspens on was extracted w th 45 ml of chloroform methanol (1) (v) (Folch et al 1957) ontaining 0 1 mg ml of butylated hydroxytoluene (BHT) as an ant o idant (Wren a d Sczcepanowska 1964) for 30 m n at room temperature The extract was washed with 9 ml of 0 6 NaCl and was l ft t 4 C o er n ght The lower phase was collected and 33 ml of a theoretical mixture of lower phase was add d to the upper

phase After 5 h at 4 C the lower phase was combined with the previous one After evaporation the residue of the lower phase was dissolved in 2 ml of methanol and the residue of the upper phase in 2 ml of distilled water The radioactivity was measured in both phases

Thin layer chromatography (t l c)

Glass plates (0 ?0 cm) were coated with a suspension of 40 g of Silica Gel H (E Merck) in 90 ml of 1 mM Na_2CO dried and then activated at 110 C for 30 min Extracted lipids (lower phase) were dissolved in chloroform and applied to a silicic acid column (? g 100 mesh Mallinckrodt) with a diameter of 1 cm 2 fractions were collected chloroform 25 ml and methanol 25 ml After evaporation of the methanol the residue was dissolved in a small volume (0 06–0 1 ml) of methanol and applied to a t l c —plate 75 μg amounts of reference phospholipids in 15 μl of methanol were also applied The solvent system used was chloroform methanol acetic acid water 50 30 8 4 (v/v) (Skipsky *et al* 1963) containing BHT 0 1 mg ml The chromatoplates were developed until the solvent front had reached 15 cm from the origin The spots were detected with iodine vapor The chromatoplates were divided into 1 cm zones and the silica gel was scraped off into liquid scintillation vials The phospholipids were extracted from the silica gel with 0 5 ml of methanol before scintillation fluid was added The recovery was about 80

Determination of radioactivity

Half a ml of the samples were transferred to vials and 10 ml of Insta Gel (for aqueous samples) or 10 ml of toluene PPO POPOP (for methanol samples) was added Packard Tri Carb liquid scintillation spectrometer (model 3375) was used The efficiency of the counting was estimated by using automatic external standardisation and curves for quenching. The incorporation of precursors into phospholipids was calculated by determining the radioactivity in the lower phase of the Folch extraction procedure The incorporation was calculated as picomoles per million cells

Determination of lipid phosphorus

Lipid phosphorus was determined according to the method of Bartlett (1959)

Statistical method

The Student s t test was used for paired comparisons

Results

Distribution of radioactivity

The radioactivity was measured in both the upper (polar) and lower phases after the extraction of lipids from mast cells incubated with labelled precursors The recovery of reference phosphatidylcholine and lysophosphatidylcholine in the lower phase averaged 96% as measured by determination of lipid phosphorus Most of the label from incubations with ^{14}C serine C-ethanolamine or ^{3}H inositol and mast cells was recovered from the upper phase and only 21 5 and 9 per cent respectively from the lower phase (Table I) In contrast as much as 56 per cent of the ^{14}C-choline added to mast cells was found in the lower phase

T l c of the extracted phospholipids

The ^{14}C-choline was found to be associated with a compound with the t l c mobility of phosphatidylcholine (R_F 0 64) and to a lesser extent (1%) with that of lysophosphatidylcholine (R_F = 0 17) with no detectable activity in the sphingomyelin area (R_F = 0 30) ^{3}H inositol and ^{14}C-serine were likewise found to be incorporated essentially into the corresponding phospholipids i e phosphatidylinositol (R_F = 0 83) and phosphatidylserine (R_F =

TABLE I Distribution of radioactivity after lipid extraction of mast cells incubated for 30 min with labelled phospholipid precursors.

Precursor	c p m per million cells[a]	
	Upper phase	Lower phase
^{1}C choline	18 530 ± 610 (44)	40 0 ± 2 610 (56)
^{1}C serine	21 000 ± 1 860 (77)	6 090 ± 240 (23)
^{14}C ethanolamine	55 690 ± 3 640 (95)	2 970 ± 130 (5)
^{3}H inositol	1 310 ± 50 (91)	130 ± 5 (9)

Means and standard errors (n = 4) are given
The distribution in per cent are given within brackets

0 90) Most of the ^{1}C-ethanolamine was associated with phosphatidylethanolamine (R_F = 0 95) but a minor amount (1 %) of the label was recovered from an area with the same R_F-value as lysophosphatidylethanolamine (R_F = 0 55)

Time course

The incorporation of the different precursors was followed for 2 h (Fig 1) By that time the incorporation of ^{1}C-choline started to level off whereas the incorporation of ^{14}C ethanolamine and ^{3}H inositol continued in an essentially undiminished manner In contrast the uptake of ^{14}C serine had reached its maximum level already after 1 h Table II shows the incorporation of the different precursors after 30 min

Effects of precursor concentration

Different amounts of unlabelled precursor were added to the incubation medium containing a constant amount of the labelled material (Fig 2) The incorporation of ^{14}C-choline increased by 99 per cent when the concentration of choline was raised from 0 07 mM to 2 07 mM The uptake of ^{14}C serine increased to a similar extent (51 %) by increasing the concentration of the unlabelled precursor In contrast there was a linear relationship between the concentration of ethanolamine added and the incorporation of C-ethanolamine The uptake increased by 575 % when 2 mM of unlabelled ethanolamine was added It was not possible to make a corresponding dilution of labelled material with myo-inositol because

TABLE II Incorporation of phospholipid precursors after 30 min incubation

Precursor	nmoles/10^{6} cells added	pmoles/10^{6} cells incorporated	% incorporation
^{14}C-choline	30	288 7 ± 36	1 3
C serine	18 6	7 6 ± 3.5	0 4
C-ethanolamine	41 4	69 1 ± 3 8	0
H inositol	1 2	0 1 ± 0 01	0 0

Means and standard errors (n = 4) are given

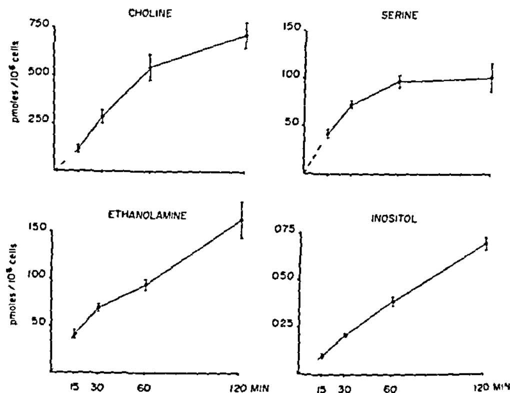


Fig 1 Time course of the incorporation of labelled precursors ^{14}C-choline (70 μM) ^{14}C ethanolamine (130 μM) and ^{3}H inositol (5 μM) Means and standard errors (n = 4) are given

the net incorporation of ^{3}H inositol (cpm) was just above the background value even though 10 μCi was added

Effect of Ca and Mg

In this series of experiments the incubation medium consisted of isotonic saline solution containing 10 % Sorensens phosphate buffer albumin (1 mg ml) and glucose (1 mg ml) On addition of 1 mM Ca^{2+} and Mg to the medium the incorporation of all precursors increased (Table III) Only in the experiments with ^{3}H inositol did 1 mM Ca^{2+} or 1 mM Mg alone significantly promote the incorporation of the precursor

Effect of antimycin A

Mast cells were preincubated for 5 min with antimycin A (1 μM) in a glucose free medium The labelled precursor was added and the incubation was continued for 30 min Antimycin A was found to inhibit significantly the incorporation of ^{14}C-choline ^{14}C serine and ^{3}H inositol (*Table IV*) *Addition of glucose (1 mM) to the medium i e* preincubated together with antimycin A partially counteracted the inhibitory effect of antimycin A (1 μM) as studied for the ^{14}C-choline incorporation Thus the inhibition was 47% and 90% respec

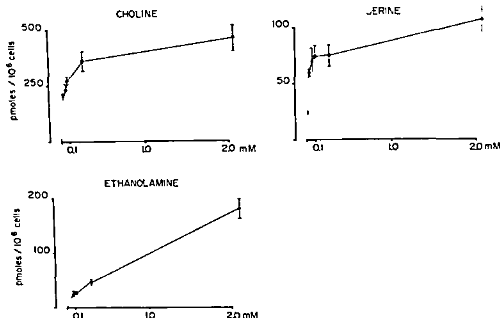


Fig 2 Effect of precursor concentration on the incorporation D fferent amounts of unlabelled precursor were added to 2 / Ci of labelled precursor Incubat on time was 30 min

tively with or without glucose present. The presence of glucose (1 mM) by itself was found to significantly stimulate the incorporation of ¹ C-choline

Discussion

A great number of studies have been published on the incorporation of phospholipid pre cursors in different systems *e g* in rat liver subcellular particles *in vitro* (Kennedy and Weiss 1956 Dils and Hubscher 1961 Bygrave and Bucher 1968 Bygrave and Kaiser 1968 Bjerve 1973) and *in vivo* (Spitzer *et al* 1969 Bjerve and Bremer 1969 Treble *et al* 1970)

TABLE III Effect of Ca² (1 mM) and Mg (1 mM) on the incorporation of lipid precursors Incubation time was 30 m n

Precursor	pmoles/10⁶ cells			
	Control	Ca	Mg³	Ca and Mg²
¹ C-choline	559 ± 0	709 ± 30 4	55 ± 5 0	97 ± 4 5
¹ C serine	53 4 ± 5 4	70 9 ± 7 9	50 1 ± 3 5	78 6 ± 10 0
¹ C-ethanolamine	36 8 ± 3 6	4 0 ± 5 5	39 3 ± 4 4	59 8 ± 4 4
H inositol	0 06 ± 0 01	0 13 ± 0 0	0 15 ± 0 03	0 1 ± 0 0

Means and standard errors (n = 4) are given
0 01 < p 0 05 0 001 < p < 0 01 p < 0 001 as compared to control values.

TABLE IV Effect of antimycin A (1 μM) on the incorporation of phospholipid precursors

Precursor	pmoles/10⁶ cells[a]			
	Control	Antimycin A	Antimycin A + glucose (1 mM)	Glucose (1 mM)
^{14}C-choline	115.9 ± 9.5 (10)	4.6 ± 1.4 (10)	61.3 ± 6.8 (6)	184.3 ± [illegible]0.0 (6)
^{14}C serine	55.5 ± 4.0 (4)	14.4 ± 1.3 (4)		
^{14}C-ethanolamine	55.4 ± 6.9 (4)	36.3 ± 6.2 (4)		
^{3}H inositol	0.17 ± 0.0[illegible] (4)	0.04 ± 0.0[illegible] (4)		

[a] *Means and standard errors are given Figure within brackets represent number of determinations*
0.001 < p < 0.0[illegible] p < 0.001 as compared to control values.

in chick brain microsomes (Porcellati *et al* 1971) in rat brain synaptosomes (Abdel Latif and Smith 1972) and in neoplastic mast cells (Pasternak and Bergeron 1970) Several of the authors reported a Ca^{++} stimulated uptake of choline serine and ethanolamine via base exchange mechanisms (Dils and Hubscher 1961 Porcellati *et al* 1971 Abdel Latif and Smith 1972, Bjerve 1973) In contrast the uptake of ^{3}H inositol in rat brain synaptosomes is inhibited by 1 mM Ca^{2+} and stimulated by Mg^{2+} and CTP suggesting that in this system the pathway for the incorporation of inositol is not by exchange (Abdel Latif *et al* 1973) The pathway for the incorporation of choline described by Kennedy and Weiss (1956) including phosphorylcholine and CDP-choline as intermediates for the incorporation of choline into phosphatidylcholine requires Mg^{2+} and CTP The cholinephosphotransferase which transfers the phosphorylcholine moiety from CDP-choline to the diglyceride is inhibited by Ca (about 1 mM) (Kennedy and Weiss 1956 Dils and Hubscher 1961 Hill and Lands 1971) In our experiments only the incorporation of inositol was significantly stimulated by 1 mM Ca^{2+} or 1 mM Mg^{2+} Thus under the present conditions our data do not suggest a major role for base exchange with regard to the incorporation of choline serine and ethanolamine in rat mast cells For choline and serine this view is further corroborated by the findings that in rat brain synaptosomes the incorporation by base exchange does not seem to be dependent on energy yielding processes (Abdel Latif and Smith 1972) Thus in this preparation it was not blocked by metabolic inhibitors such as 2,4-dinitrophenol In contrast we found that the incorporation of choline serine and inositol was inhibited by antimycin A This agent inhibits the electron transport between cytochrome b and c in the mitochondrial respiratory chain (for references see Wainio 1970) The observation that glucose partially counteracted the inhibitory effect of antimycin A on the incorporation of choline into the mast cells indicates that glycolysis to some extent can supply the energy required for this process

The incorporation of ethanolamine differed from that of the other precursors studied Thus it was linear during the experimental period and it was neither saturated by increasing the concentration from 0.07 mM to 2.07 mM nor blocked by antimycin A This indicates that ethanolamine is transported into the cells via diffusion Whether the energy dependency of the incorporation indicated for the other precursors reflects an active transport or energy requiring steps in the synthesis of the phospholipids can not be distinguished It must be

recognized that our system involves intact cells in contrast to the cited studies on microsomes or mitochondria It may be recalled that the uptake of *e g* choline into human blood platelets has been found to be energy requiring *i e* an active transport process has been indicated (Green *et al* 1972)

The present study was principally undertaken to establish conditions for the incorporation of labelled precursors into rat mast cell phospholipids This would set the conditions for an analysis whether changes in the phospholipid composition occur on histamine release from rat mast cells From preliminary experiments it is clear that significant radioactivity appears in the incubates when mast cells labelled with ^{14}C-choline ^{14}C serine or ^{14}C-ethanolamine are exposed to the histamine liberators n-decylamine and compound 48/80 Predominantly the base is recovered but also the corresponding phospholipids and lyso-compounds have been identified

There are previous indications of an increased lipid turnover on histamine release from mast cells Thus biologically active lipid soluble principles are formed on histamine release from isolated rat mast cells induced by compound 48/80 indicating hydrolysis of membrane lipids (Uvnäs and Thon 1959 Änggård *et al* 1963) Part of the biological activity recovered from the experiments with isolated mast cells (Änggård *et al* 1963) had characteristics similar to those of the prostaglandins Hydrolysis of fatty acids esterified to phospholipids is regarded as the initial step in the biosynthesis of the prostaglandins (Lands and Samuelsson 1968) In fact it has been postulated that the activation of a phospholipase A like enzyme in the mast cell is an essential link in the histamine release process (Hogberg and Uvnäs 1957) Since isolated rat mast cells can incorporate phospholipid precursors we consider this system a suitable model to study the turnover of phospholipids during a release process Such studies might have significance for the reactions of other secretory cells Thus the histamine release and the related exocytosis triggered by agents such as antigen and compound 48/80 seems to operate via mechanisms similar to those activated when *e g* insulin and adrenal medullary amines are released (for references see Becker and Henson 1973)

This work was supported by grants from the Swedish Medical Research Council (project No K73 14X 3774) and Magnus Bergvall s foundation

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Acta physiol scand 1975 94 63–73

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Selective Localization of Histamine to Electron Dense Granules in Antigen-Challenged Sensitized Rat Mast Cells and to Similar Granules Isolated from Sonicated Mast Cells

An Electron Microscope Autoradiographic Study

By

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Received 6 December 1974

Abstract

ANDERSON P and B UVNÄS *Selective localization of histamine to electron dense granules in antigen challenged sensitized rat mast cells and to similar granules isolated from sonicated mast cells An electron microscope autoradiographic study* Acta physiol scand 1975 *94* 63–73

The subcellular localization of histamine was studied in sensitized rat mast cells following antigen challenge and in granules obtained from sonicated cells using an electron microscope autoradiographic technique The mast cells were furnished with labelled histamine by incubation in ³H histidine The silver grain distribution (reflecting the localization of radioactive histamine) was highly non random The highest silver grain densities occurred over homogeneous electron dense (normal) granules and moderately electron dense granules Swollen less electron dense (changed) granules with a reticular appearance and devoid of a limiting membrane had the lowest density of all subcellular structures studied and were therefore probably almost free of histamine There was a good correlation between the percentage of electron dense granules the histamine content and the silver grain density in saline washed granule fractions isolated after sonication of mast cells for different times These results support the hypothesis that histamine release occurs during the sequential exocytosis of storage granules and during the sonication of mast cells probably as a cation exchange between the amine which is ionically bound to the heparin protein complex of the granule matrix and cations from the extracellular fluid The exchange will occur as soon as the perigranular membrane becomes permeable to water and cations

When mast cells are treated with a histamine releaser such as compound 48/80 or when sensitized mast cells are challenged with antigen *in vitro* characteristic ultrastructural changes occur in the cells during the histamine release process (Bloom and Haegermark 1965 Singleton and Clark 1965 Rohlich Anderson and Uvnäs 1971 Bloom and Chakravarty 1970 Anderson Slorach and Uvnäs 1973) Some of the granules which normally have a homogeneous electron dense (osmiophilic) appearance acquire a swollen less electron dense reticular appearance These changed granules which are found both outside and inside the cells are also devoid of a surrounding trilaminar membrane By using extracellular tracer substances such as lanthanum or hemoglobin it has been established that all changed granules even those lying deep inside the cell in seemingly isolated pockets with no a

parent communication with the exterior, were in fact located in labyrinthic cavities formed during the release process and were in communication with the extracellular milieu (Röhlich *et al* 1971 Anderson *et al* 1973) This finding was in agreement with the hypothesis of Uvnäs that the histamine release from all changed granules can be explained as a simple cation exchange between histamine bound to the heparin protein complex and cations in the extracellular fluid (Uvnäs and Thon 1967) Accordingly the histamine remaining in mast cells after exocytosis should be localized to the unchanged electron dense granules

Isolation of granules from sonicated mast cells yields a mixed population of homogeneous *electron dense granules and swollen less electron dense granules with a reticular appearance* the former with a trilaminar membrane the latter devoid of a limiting membrane On washing in saline the granules release part of their histamine to the suspension medium the higher the proportion of changed granules the greater the release (Anderson *et al* 1974)

The present electron microscope autoradiographic study demonstrates in a direct way the subcellular localization of histamine in both antigen-challenged sensitized mast cells and in saline washed granules obtained from sonicated mast cells To be able to localize the histamine autoradiographically the granules were furnished with ^3H histamine by incubating the mast cells *in vitro* with ^3H histidine

Methods

Sensitization procedure and preparation of cell suspension

Male Sprague Dawley rats (180 g) were sensitized by s.c. injection of [illegible] 0.5 ml of egg albumin solution (100 mg ml) and [illegible] 0.5 ml of pertussis vaccine as adjuvant 3 weeks later a mixed cell suspension was collected from the peritoneum and pleura as described by Anderson *et al* (1973) using a salt solution (NaCl 130 mM CaCl$_2$ [illegible]0 mM) buffered with 10 [illegible] v v Sørensen buffer (Na$_2$HPO$_4$ KH$_2$PO$_4$ 67 mM) pH 6.7 and containing 0.1 mg of human serum albumin per ml

Incubation with ^3H histidine

The mixed cell suspension was centrifuged ([illegible]00 g 5 min [illegible] C) and the resulting sediment was then incubated for [illegible] h at 37 C in [illegible] ml of the above mentioned buffered salt solution containing 1 mCi ^3H histidine ([illegible] histidine [illegible] ^3H Spec act 55 Ci mmol) giving a final histidine concentration of 0.86 [illegible] 10^{-6} M The incubation was stopped by adding 8 ml of the salt solution ([illegible] C) and the cells were washed twice before being resuspended in the salt solution to give a concentration of 0.7 [illegible] 10^6 mast cells per ml

Incubation with antigen

Samples for electron microscope autoradiography and for histamine assays were run in parallel

0.9 ml aliquots of the mixed cell suspension were preincubated at [illegible]5 C for 5 min before adding 5 [illegible] of egg albumin dissolved in 0.1 ml of the salt solution controls were incubated with 0.1 ml of the salt solution Incubation was stopped after different times (30 60 90 180 or 600 s) by adding either 9 ml of cold (4 C) [illegible] glutaraldehyde in [illegible] M Millonig phosphate buffer (NaH$_2$PO$_4$ H$_2$O 134 mM NaOH 107 mM) pH = 7.3 (electron microscope autoradiography) or 9 ml of ice cold buffered salt solution (histamine assays)

Electron microscope autoradiography of cells and material

The samples were fixed for 5 10 min at 4 C and then for 1 h at [illegible] C The cells were washed postfixed in 1 [illegible] OsO$_4$ in Millonig phosphate buffer (45 min 4 C) contrastained with 0.5 [illegible] uranyl acetate in acetate buffer pH = 5.0 (1 h [illegible] C) suspended in a 1 [illegible] gelatin solution dehydrated in an ethanol series, soaked in propylene oxide and embedded in Araldite (for a detailed description of the procedure see Anderson *et al* 1973)

Thin sections with a grey interference colour (less than 600 Å thickness according to Williams and Meek 1966) were cut on a Reichert ultramicrotome OmU3 (C Reichert Optische Werke A G Austria) The

sections were picked up on Formvar-carbon-coated 00 mesh copper grids (VECO Zeefplatenfabriek B V Eerbeek Holland) The grids with sections were attached with double coated tape (Scotch No 465 1/2 3 M Co St Paul, Minn) to microscope slides

Emulsion preparation and coating were performed by a modification of the technique described by Caro and Van Tubergen (196) and Caro (1969) *Ilford L4 emulsion was melted at 45 C and diluted* with an equal volume of distilled water The emulsion was cooled to 30 C. Emulsion films were prepared by dipping a copper wire loop (inner diameter 4 cm) into the emulsion After the film had gelled in the air as shown by the change in the interference colour in reflected red light it was applied to the microscope slide carrying the grids The emulsion films applied to the grids were shown to consist of a monolayer of silver halide crystals as judged from the interference colour in white light and checked by electron microscopy after every coating session

Since no major difficulties were experienced in obtaining even monolayers of silver halide crystals *from our Ilford L4 emulsion this technique was used instead of for example the more laborious "dipping"* technique of Salpeter and Bachmann (1964) in which the monolayer is formed on a flat substrate provided by a microscope slide

The emulsion-coated sections were then exposed for 2 8 weeks at 4 C in sealed light tight plastic boxes containing Drierite They were then developed according to Caro and Van Tubergen (196_) at _0 C in Kodak Microdol X diluted with 3 vol. of distilled water for 5 min washed in 1% acetic acid for 10 s fixed in Kodafix diluted with 3 vol of distilled water for 5 min washed in running tap water (5 min) and finally washed in distilled water (1 min) The grids were then separated from the microscope slides dried and stained with 2 aqueous uranyl acetate *for _0 min at 60 C, followed* by lead citrate *for 15 min*

Before the autoradiographs were analyzed it was confirmed that the grain pattern was not due to "back ground or to positive chemography (production of a latent image by direct chem cal action) by making autoradiographs of similarly [illegible]pared but unlabelled material. Negative chemography (eradication of the latent image by direct ch[illegible]mical action) was checked by observing the beha iour of fogged emulsion layers overlying the secti[illegible]s

Since no chemical i raction was found between the sections and the emulsion in our material we did not cover the sec[illegible]is with a layer of carbon by vacuum evaporation before applying the emulsion as sug [illegible]oehler *et al* (1963) Bachmann and Salpeter (1964) and Salpeter and Bachmann (1964) According to Salpeter and Bachmann (1964 and 1972) this intermediate carbon layer is not essential for protecting the sensitivity of the more stable Ilford L4 emulsion as it is for other nuclear emulsions

The sections were observed in a Philips EM 300 electron microscope at 80 kV Semi thin sections for light microscopy were stained with a solution of toluidine blue and azur A containing 60% sucrose

P eparation of mast cell suspens n in ubation with ^{3}H h st d ne and isolat on of g anules

Mast cells were obtained from the peritoneal and pleural cavities of 10–12 male Sprague Dawley rats (350–400 g) *using a buffered salt solution (NaCl 145 mM KCl 7 mM CaCl 0 9 mM with 10 v/v* Sörensen buffer (Na HPO + KH_2PO_4 67 mM pH = 7 0) a d isolated by density gradient centrifugation in Ficoll as described by Anderson *et al* (1974) The cell suspension containing more than 95 mast cells was incubated with ^{3}H histidine as described above for mixed cells After washing the isolated mast cells were resuspended in 25 ml of ice cold 0 34 M sucrose 5 ml aliquots cooled in ice were sonicated for 5 or 1 0 s with a MSE 100 W ultrasonic disintegrator (MSE Ltd., London England) set a 4 / m amplitude using a 10 mm probe The sonicated suspensions were centrifuged (350 g 15 min 4 C) and the granul containing supernatants were then recentr fuged (3 000 × g 20 min 4 C) The granule sed ments thus obtained were suspended in 0 15 M NaCl adjusted to pH 7 0 with Sörensen phosphate buffer and half of the suspens on was taken for electron microscope autorad ography and the other half for histam ne heparin and prote n assays The samples were centrifuged (3 000 g _0 min 4 C) and the supernatants were d scarded

El ct on microscope auto ad og aphy of solated mast ll g anules

The granule pellets were fi ed with cold (4 C) 2 5 glutaraldehyde n Millon g phosphate buffer pH = 7 3 for 40 m n at room temperature and the granule suspens ons were then centrifuged (3 000 × g 0 min 20 C) The granules were washed with Millonig phosphate buffer pH = 7 3 and postfi ed in 1 OsO i the same buffer for 40 m n at 4 C. The granules were then centrifuged down (3 000 g 0 m n 4 C) before be ng dehydrated in an ethanol series, transferr d through propylene oxide and embedded in Araldite

Thin sectioning, emulsion coating, exposure, development and staining were all carried out as described above for sensitized mast cells.

Confirmation that histamine was the only significant radioactive compound in mast cells incubated in ^{3}H histidine

A pure mast cell suspension was obtained from the peritoneal and pleural cavities of 1[illegible] male Sprague Dawley rats by density gradient centrifugation in Ficoll as described above under "Preparation of mast cell suspension". A mixed cell suspension was collected as described above under "Preparation of cell suspension". The cell suspensions were incubated with ^{3}H histidine (1 mCi. Spec. act. 55 mCi mmol, final histidine conc., 0.9×10^{-4} M) in 2 ml of buffer for 1 h at 37 C. After washing, the cell sediments were suspended in 1 ml of buffer. 100 μl of 3 N HCl was added and the cells were heated at 80 C for 5 min. 10 μl of this suspension was taken for ascending paper chromatography on Whatman no. 1 paper marked out in strips 4 cm wide. The following solvent system was used (all ratios by volume): n butanol : pyridine : water 1 : 1 : 1. After drying, the chromatograms were cut into 1 cm lengths and, after cutting into smaller pieces, these were placed in counting vials together with 10 ml of Instagel scintillation liquid (Packard Instrument Co., Inc., La Grange, Ill. USA) and the radioactivity was measured in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc.). All results were corrected for quenching and were calculated as disintegrations per minute (DPM). Reference chromatograms for histidine and histamine were run using either ^{3}H or ^{14}C labelled compounds or using non isotopic substances and then staining with ninhydrin. The radiochemical purity of the isotopic substances used was also checked by paper chromatography.

Histamine, heparin and protein assays

Histamine was assayed by the method of Shore, Burkhalter and Cohn (1959) as modified by Bergendorff and Uvnäs (197[illegible]). Heparin was measured by the amino-sugar method of Cessi and Piliego (1960) and is expressed in terms of a heparin standard. Protein was determined by the method of Lowry *et al.* (1951).

Histamine release is expressed as the percentage of the total histamine in the antigen treated sensitized cells. Histamine, heparin and protein were calculated as μg/sample in the isolated granule preparations and the results are presented as the histamine/heparin and histamine/protein ratios.

Method of analysis of electron microscope autoradiographs

A large number of sensitized mast cells, both antigen treated and control cells, were photographed in the electron microscope. The e.m. plates obtained were printed on photographic paper at a final magnification of 70 000 times. Analysis of the silver grain distribution and the grain densities was performed on [illegible] from 10 mast cells treated with antigen for 10 min at [illegible]5 C. The exposure time for these cells was 4 weeks.

The method of analysis followed essentially that described by Williams (19[illegible]9). Each silver grain on the micrographs was circumscribed by a circle, the size of which was chosen to equal the "resolution" of our system, as defined by Bachmann and Salpeter (196[illegible]), i.e. the circle had 50 [illegible] probability of enclosing the site of the disintegration. Using Ilford L 4 emulsion and assuming a section thickness of [illegible]00 Å, the circle has a radius of approximately 1 [illegible]50 Å according to Bachmann and Salpeter (1965).

Before starting the analysis of the tissue present in these 50 [illegible] probability circles, the different ultrastructural items of interest to be found in the mast cells were listed. Dense granules, moderately dense granules, less dense granules, cytoplasm and nucleus were considered as "single" entities (i.e. grains could be localized completely inside them). "Junctional" items were combinations of the above mentioned ultrastructural entities with cytoplasm or combinations of "single" entities with cytoplasm and nucleus. [illegible] compound items (i.e. groups of ultrastructural items smaller than the circle diameter together with whatever surrounding material is included within the circle) were also defined. [illegible] many of combinations of plasma membrane and "single" items or plasma membrane and "junctional" items.

Using the above classification, the 110 silver grains obtained from our material fell into 19 different grain categories.

To find the relative volume of each tissue component (item), the effective area of each item was calculated by the application of circles scored on a clear transparent "overhead" film with [illegible] over the micrographs of the mast cells, [illegible] circles, 5 mm diameter corresponding to 50 [illegible] probability circles at a magnification of 70 000 times.

To determine if the silver grain distribution was random or non-random, a comparison was made between the circle and the grain frequency distributions using a χ^2 test. The frequency distribution of circles

over the various ultrastructural features is an estimate of the frequencies of silver grains to be expected over those features if the grains were scattered randomly)

The specific activities that is the number of silver grains per unit area feature (silver grains/ circles) were calculated for the different ultrastructural items in the mast cells.

Electron microscope autoradiographs of granules isolated from mast cells incubated in H histidine and then sonicated were analyzed using the same procedure except that the specific activities were expressed in silver grains per granule

Materials

Araldite (Durcopan ACM) FLUKA AG Buchs SG Switzerland Egg albumin Difco Laboratories Inc Detroit Michigan USA Ficoll AB Pharmacia Uppsala, Sweden Glutaraldehyde (25% especially purified for electron microscopy) TAAB Laboratories Reading, England Heparin sod um (pig mucous) containing 9 2 per cent sulphur AB Vitrum Stockholm Sweden Human serum albumin (free from preservatives) AB Kabi Stockholm Sweden Ilford L 4 emulsion Ilford Ltd Essex, England Pertussis vaccine (0×10^{9} bact per ml) SBL, Stockholm Sweden L-(_5 ^{3}H histidine), Spec act 55 Ci mmol (Batch 9) and 58 Ci/mmol (Batch 27) and histamine (ring ^{14}C) Spec act 59 mCi/mmol Amersham England All other substances were obtained from the usual commercial sources

Results

Fate of the radioactivity during preparation of the cell suspension for electron microscopy

In order to determine possible extraction of the isotope during preparation for electron microscopy the radioactivity in the solutions used at the different steps in the preparation procedure was measured in three experiments with mixed cell suspensions After glutaraldehyde fixation and washing, not more than 0.2-1 6% of the total radioactivity was lost during osmium tetroxide fixation washing ethanol dehydration and propylene oxide soaking Thus almost all the radioactivity taken up by the cells during the H histidine incubation was still present in the cells when embedded in Araldite

Fate of ^{3}H histidine in pure mast cell suspension

When a pure mast cell suspension was incubated with H histidine (1 mCi Spec act 55 Ci/mmol histidine conc $0\,9 \times 10^{-5}$ M $4\,5 \times 10$ mast cells/ml) for 1 h at 37 C using the same method as used for sensitized mixed cell suspensions prior to em autoradiography more than 90 (91% –96) of the radioactivity was found to be H histamine as shown by paper chromatography

Sensiti ed mast cells treated with antigen

Histamine release When ^{3}H histidine incubated mast cells in a mixed cell suspension from peritoneum and pleura from sensitized rats were challenged with antigen (5 μg egg albumin/ml) for different times at 25 C the histamine release started after a latent period of 30 s increased to 14 % after 3 min and reached a maximal value of 16 after 10 min in the present em autoradiography experiment

Morphology The changes observed in the morphology of sensitized mast cells during antigen induced histamine release have been described in detail earlier both at the light and at the electron microscopic level (Anderson *et al* 1973) and are therefore omitted It is sufficient to point out that when viewed in the light microscope cells treated for longer times contain more and more granules which stain pink with a toluidine blue azur A

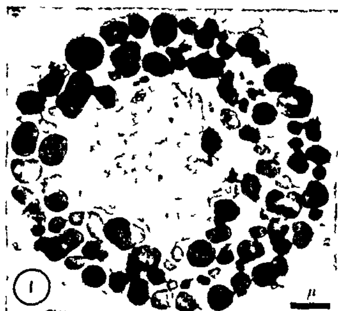


Fig 1 Sensitized mast cell treated with antigen for 10 min at 5 C. The cytoplasm is filled with homogeneous electron dense granules. No signs of any ultrastructural changes can be seen in this cell. Most silver grains are found over the dense granules Magnification ×9 500

tion instead of dark blue the colour associated with granules in untreated cells In the electron microscope these changed granules had a swollen less electron dense reticular appearance in contrast to the homogeneous electron dense appearance of normal granules in unchallenged cells

Grain distribution Silver grains were found over both the mast cells and the leucocytes in our mixed cell suspension In the mast cells most grains were found over the electron dense granules but some were also found outside the granules some over the nucleus (Fig. 1 and Fig 2)

To establish if the grain distribution was random or non random a comparison between the grain distribution and the circle distribution was made using a χ^2 test The grain distribution was found to be highly non random (χ^2 - 118 07 P - 0 001) which is also evident from Table I

The silver grain densities of the different subcellular structures (items) in the mast cells are seen in Table I Dense (normal) granules had the highest grain density (% silver grains/ % circles) (3 87) followed by moderately dense granules (2 85) while less dense (changed) granules showed the lowest density (0 13) Junctional items such as dense granules cytoplasm and moderately dense granules cytoplasm had grain densities between those of the corresponding single items

The ratios between the silver grain densities of different subcellular structures are shown in Table II The ratio between dense granules and less dense granules was found to be 29 77 which means that dense granules contain about 30 times more radioactivity than less dense granules Moderatel dense granules contained about 20 times more radioactivity than less dense granules. Dense granules contained 3 5 times more label than cytoplasm or nucleus while less dense granules had only 1/9 or 1 (of the label of the cytoplasm or the nu leus



Fig 2 Sens tized mast cell treated with antigen for 10 min at 25 C. Many "changed swollen less electron dense granules lying in ca ities in the cell can be observed Some granules (X) have an elec tron density between that of the normal electron dense granule and that of the changed less elec t on dense granule S lver grains are mainly found over dense granules less electron dense gran ules are almost de o d of grains Magn fication ×9 300

Granules isolated from sonicated mast cells

Pure granule fractions were obtained from ^{3}H histidine incubated mast cells by sonication at 4 μm amplitude for either 5 or 120 s

Morphology Two types of granule were found One type was homogeneous electron dense and surrounded by a trilaminar membrane the other type was less electron dense and had a swollen reticularnet like structure and was devoid of a limiting unit membrane (Fig 3)

TABLE I Analysis of the grain distribut on on em autoradiographs of sensitized rat mast cells incubated with H histidine prior to inducing histamine release by antigen challenge for 10 min at 25 C.

Item	sil er grains	circles	silver grains/ circles
Dense granules	35 65	9.22	3 87
Moderately dense granules	74	0 96	2 85
Less dense granules	3 87	29 70	0 13
Nucleus	10 3?	12 79	0 81
Cytoplasm	7 4	6 53	1 14
Dense granules Cytoplasm	9 84	19 40	1 54
M d granules/Cytoplasm	1 94	0 84	31
L. d granules Cytoplasm	10	5 53	0 38
Dense granules/Nucleus Cytoplasm	1 94	1 40	1 39
Other items	4 18	13 63	0.31
	(310 sil er grains)	(495 circles)	

Comparison of the silver grain d stribution with the circle d stribution $\chi^2 = 118.07$ $P < 0.001$ *i.e* the grain distribut on is highly non random



Fig. 3. High power em autoradiograph of granules obtained by sonication of ^{3}H histidine incubated mast cells at 4 μm amplitude for 5 s. Note that the electron dense granules are surrounded by a trilaminar membrane that can be seen at favourable planes of sectioning (arrows). No membrane can be seen around changed, less electron dense granules. Magnification × 30 000.

At the shorter sonication time 51% of the granules were found to be dense granules compared to 17% at the longer sonication time.

Histamine content. The histamine/heparin and histamine/protein ratios were found to be higher at the shorter sonication time (0.11 and 0.15 respectively, means of 4 samples taken in parallel with the em autoradiography) than at the longer time, when both ratios had decreased to 0.04.

Grain distribution. The silver grain distribution was analyzed in 7 autoradiographs taken from the granule fraction obtained after 5 s sonication and from as many prints from the experiment with 120 s sonication. 213 silver grains were found in totally 1 544 granules. At 5 s sonication time the ratio for grains per granule (grain density) was found to be 0.?0 and at 120 s sonication time the ratio had decreased to 0.07 (Fig. 4). The silver grain density for dense granules was 0.33 and for less dense granules 0.05 (Table III).

TABLE II. Analysis of the ratio between the grain density of different items on em autoradiographs of sensitized rat mast cells incubated with ^{3}H histidine prior to inducing histamine release by antigen challenge for 10 min at 37°C.

	Density ratio	Sample estimates of the standard deviation		t value	df	P
Dense granules / Less dense granules	[illegible]	5.91	0.10	[illegible]	11	0.05
Dense granules / Moderately dense granules	[illegible]	5.91	1.85	1.03	11	N.S.
Dense granules / Nucleus	[illegible]	5.91	0.51	3.04	18	0.01
Dense granules / Cytoplasm	[illegible]	[illegible]	1.85	[illegible]	17	0.05
Moderately dense granules / Less dense granules	[illegible]	1.85	0.10	3.5	6	[illegible]
Moderately dense granules / Nucleus	[illegible]	1.85	0.51	3.39	11	0.01
Moderately dense granules / Cytoplasm	[illegible]	1.85	1.85	0.85	10	N.S.
Less dense granules / Nucleus	[illegible]	0.10	0.51	[illegible]	13	[illegible]
Less dense granules / Cytoplasm	[illegible]	0.10	1.85	0	[illegible]	[illegible]

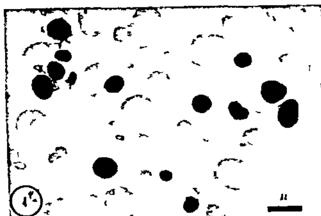


Fig 4 Em autoradiograph of a pure granule fraction obtained from ³H histidine incubated mast cells sonicated at 4 μm for 1 0 s The silver grains are mostly associated with the electron dense granules Magnification ×8 800

Discussion

A mixed cell suspension was used in the present electron microscope autoradiographic study instead of isolating the mast cells on a Ficoll gradient The time of handling the cells prior to fixation could therefore be shortened.

When a pure mast cell suspension was incubated with ³H histidine more than 90% of the radioactivity was found to be present as histamine Similar findings have been reported by Cabut and Haegermark (1968) At least 90 % of the silver grains overlying the mast cells in our cell suspensions must therefore be due to the presence of labelled histamine

The histamine release from sensitized mast cells increased progressively with increasing incubation time after antigen challenge Simultaneously some of the normal dark blue staining granules in the mast cells were stained pink (metachromatic) with toluidine blue azue A in the light microscope In the electron microscope these changed" granules had a swollen less electron dense reticular appearance instead of the homogeneous, electron dense feature of normal granules At the shorter incubation times there were relatively few changed granules and these were mostly located near the cell periphery The number of changed granules increased with longer incubation times and they could then be found seemingly deep inside the cells

TABLE III Means and standard deviations of the silver grain density (silver grains granule) in dense and less dense granules isolated from mast cells incubated in H histidine and sonicated at 4 μm amplitude setting for 5 and 1 0 s and t value for the differences obtained

	Mean	Sample estimate of the standard deviation	t value	df	p
Dense granules	0.33	0 10	9 1	5	<0 001
Less dense granules	0 05	0 04			

13 silver grains were analyzed in 1 544 granules on 14 autoradiographs from both sonication t

The silver grain distribution was highly non random in antigen-challenged sensitized mast cells The highest grain density reflecting the highest amount of labelled histamine was found in the electron dense (normal) granules The lowest density was detected in less dense (changed) granules Moderately dense granules which are granules with an electron density between that of normal and changed" granules were also sometimes found Similar granules described as slightly swollen granules have been observed earlier when mast cells were incubated with compound 48/80 (Röhlich *et al* 1971) and when sensitized mast cells were treated with antigen (Anderson *et al* 1973) These granules are often situated close to the extracellular space and might therefore represent granules releasing histamine during an influx of cations and water by the ion-exchange mechanism suggested by Uvnäs and collaborators

The analysis of the silver grain distribution thus clearly showed that the greater part of the radioactive histamine was localized in dense or moderately dense granules or in junc tional structures including these granules The smallest amount of histamine was found in less dense (changed) granules The fact that some grains were found outside dense and moderately dense granules in other subcellular structures can be explained as being partly to the scattered tail of the distribution curve for the silver grains and possibly partly due to the presence of a small amount of unchanged radioactive histidine The presence of labelled histidine has in fact been described in other cell types after glutaraldehyde fixation for em autoradiography (Salpeter and Bachmann 1972)

In order to compare the distribution of histamine and the ultrastructural changes in mast cell granules without interfering structures granules were isolated from ³H histidine incubated mast cells

Sonication for 5 s gave a granule fraction containing 51% electron dense granules The granule fraction had a histamine content of 0 11 when expressed as the histamine heparin ratio (see Methods for definitions of ratios) or 0 15 when expressed as the histamine protein ratio and a total grain density of 0 10

Sonication for 120 s gave a granule fraction containing 17% electron dense granules. This fraction had a granule content of histamine of 0 04 (histamine/heparin and histamine/protein ratios) and a total grain density of 0 07

Thus it is evident that there exists a good correlation between the percentage of electron dense granules the granule content of histamine and the total grain density (reflecting the amount of histamine per granule) when the granule fractions obtained after the two different sonication times are analyzed

Calculation of the grain densities for isolated dense granules (0 33) and less dense granules (0 05) showed that dense granules with an intact perigranular membrane contained at least 7 times more radioactive histamine than the less dense granules

In the present em autoradiographic investigation it was thus possible to show a selective localization of histamine to electron dense granules in antigen-challenged sensitized mast cells and to similar granules isolated from sonicated mast cells "Changed" granules on the other hand had the lowest grain density of all subcellular structures in the mast cell and could be regarded as almost free of histamine

The investigation has been supported by grants from The Swedish Medical Research Council (B74 14X 2943 06 and B75 14X 2943 074) and King Gustaf Vth s 80th Anniversary Foundation

The skilful technical assistance of Miss Anna Lena Hemmingsson is grateful acknowledged

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Robinson *et al* 1967) Since these lines of work turned out to be negative a more general screening was initiated

Part of this work was presented at a Neurosciences Research Program Work Session Boston May 1974 (Terenius 1975 a) and at the 28th Scandinavian I harmacologic Society Meeting Odense August 1974 (Terenius and Wahlstrom 1974)

Materials and Methods

Substa ces and reagents Dihydromorphine (DHM) labelled with tritium by catalytic hydrogenation of morphine at a specific activity of 55-60 Ci/mmol was obtained from New England Nuclear Corp Bos ton Mass Unlabelled DHM was prepared by the same procedure by the author The peptides for column calibration were purchased from Sigma Co St Louis Mo All other chemicals were of reagent grade

Receptor preparations from synaptic plasma membranes of rat brains were obtained as described ear lier (Terenius 1974) The receptor preparation from the guinea pig ileum also followed a previously de scribed procedure (Terenius 1973 c) These preparations were obtained on a large scale basis and aliquots were kept frozen at −70 C for up to 6 months without reduction in receptor content

The HEPES buffer had the following composition NaCl 124 mM KCl 5 mM KH PO 1 ₊ mM CaCl 0 75 mM N 2 hydroxyethyl p perazine N 2-ethanesulfonic acid HEPES 26 mM It was adjusted to pH 7 4 at 25 C

The liquid scintillation cockt il was toluene 3 1 2 butoxyethanol 60 ml PPO 15 g and dimethyl POPOP 0 9 g

Scre g of b ain extracts fo recepto aff ity A method was required that could be used for a large number of crude extracts and more or less purif ed fractions The receptor preparation had to be as free as possible from degradative enzymes A technique already described (Terenius 1974) meets these criteria (1) it utilizes the synaptic plasma membrane (SPM) fraction of rat brain from which enzymes in cytoplasm and mitochondria are removed during the processing (2) it is not very sensitive to the eventual presence of inorganic salts since the assay is run in a physiologic buffer (3) it is rapid and a large number of samples can be run simultaneously

All extracts and fractions were f rst lyophilized and then made up in HEPES buffer before the receptor assay Of the extract or fraction 0 1 ml was added to 0 3 ml of HEPES buffer with SPM (or ileum prepa ration) corresponding to about 0 4 mg of protein and labelled dihydromorphine to a final concentra tion of 0 8 × 10 M The mixture was incubated in a reciprocating incubator at 25 C for 40 min The in cubation was terminated by centrifugation in the cold for 10 min in a Microfuge (Beckman) The SPM pellet was obtained by cutting the tip of the tubes It was digested by 1 ml of Soluene (Packard) toluene (1 3 v/v) Liquid scintillation countinv of the radioactivity of the pellets and of the medium al quots fol lowed Each experimental run included the following samples

Sample	DHM H	Extract	Nonlabelled DHM (final concentration 10− M)
Control	+		−
Experimental	+	+	−
Carrier	+	−	+

Control or carrier samples conta ned 0 1 ml of HEPES buffer instead of ext act At least 3 to 4 mples of each kind or each concentration were run in an experiment

The purpose of including the carr er samples was to correct for nonspec fi (nons 1 ble) bi ding Values for carrier samples were subtracted from control a d experimental l es The ffe t f a fr ction on receptor bind ng is then obtained in percent of the corrected control binding (− 100) By the u e of a log concentration inhibit on curve which was sim lar for m ny preparatio s (F ig) the inhibition value was transformed to relati e concentrat on of receptor blocker

P epa at n of bra n ext acts (cf F g 1) Female Spr gue D wley rats weighing ab out 125 g were ex sangui ated decapitated and the whole bra n e cept the er bell m was taken into ice-cold I otter I l e hjem homogeni ers The brains were thoroughly homoge i d with two volumes of ice-col l 0 1 M buffer of pH 4 0 The homoge ate was deprotenized by heati g at 70 C fo r 20 min centrifuged

filtered through a PM10 (Amicon) filter (nominal cut off 10 000 dalton) After the heating all further purification steps were carried out at 0 – +4 C

Chromatographic parameters 20 ml of the ultrafiltrate adjusted to pH 2 9 with HCl was run on a carboxymethyl cellulose ion exchanger (CM52 Whatman 13 × 2 cm) pre equilibrated with 0 M acetic acid Stepwise elution with about 2 0 ml of 0 2 M acetic acid 150 ml of 1 M acetic acid and 200 ml of 5 M acetic acid followed The eluent was monitored in an Uvicord (LKB Beckman) at 254 nm and about 50 ml of the break through peak of 1 M acetic acid (Fraction A) and 70 ml of the corresponding peak of 5 M acetic acid (Fraction B) were lyophilized Fraction A was redissolved in 1 ml of water and run on a PGM 2000 (Merck 100 × 0 9 cm) column in water The effluent was continuously monitored in an Altex Model 150 Biochemical UV analyzer set at 254 nm (Fig 4)

Analyses Concentrations of Na^+ and K^+ were measured with an Eppendorf flame photometer against factory standards Assays for primary amines with fluorescamine (Fluram Roche) were carried out at pH 8 1 (Udenfriend *et al* 1972) A standard curve with leucine was included on each experimental occasion

Results

Initial screening of brain extracts Since the extracts were to be tested in a biologic system extreme pH or ionic strength were not allowed An ideal way to circumvent inorganic salts would be extraction into organic solvents which could be evaporated later As mentioned in the introduction a number of attempts were made to isolate receptor binding blockers in chloroform by extraction of brain homogenates at different pH s No significant receptor blocking activity was found in concentrated extracts from 5–10 rat brains When we followed the extraction scheme of Green and co workers no binding activity was found in the petroleum ether extract where they found the active substances by bioassay (Green *et al* 1963 Robinson *et al* 1967) On the other hand the water phases were slightly active in our system (and inactive in theirs) Subsequent work was directed to water soluble fractions

Water extracts were obtained as described in Methods After highspeed centrifugation ultrafiltration with different Amicon filters was applied Even if we used filters with a nominal cut off of 1 000 dalton there was no evidence of accumulation of receptor blocking factors in the retenate (concentration factor 10–20 fold) In all subsequent work a filter with a nominal cut off of 10 000 dalton was used to speed up the filtration The next step in the isolation procedure was ion exchange chromatography

Isolation of a receptor blocking endogenous factor An initial desalting step was accom

TABLE 1 Chemical characterization of the different fractions of the purification procedure Mean values from several experiments are shown The values are given per one rat brain (1 4 g wet weight)

Fraction	Absorbance units (260 nm)	Primary amine groups (meq)[a]	Na^+ (meq)	K^+ (meq)	Receptor activity (units)[b]
Extract (after ultrafiltration)	50	0 34	0 2	0 1	
Fraction A	0 57	3×10^{-5}	N D [c]	N D	0 27
Fraction C	0 021	$? 5 \times 10^{-8}$	N D	N D	0 18

[a] Fluorescamine reaction leucine equivalents
[b] For reasons given in the text receptor activity of the crude extract is not measurable Units defined as in Fig 2
[c] N D = not detectable

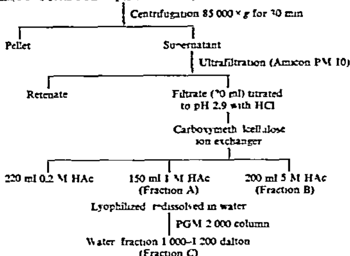


Fig. 1 Purification of an endogenous receptor blocking factor

plished by ion exchange chromatography on carboxymethyl cellulose at pH 2.9 (Fig. 1) Substances with sufficient basicity will be retained and are eluted by an increase in concentration of acid. The Fractions A (and B) are substantially free from inorganic salts (Table I) and have considerable receptor blocking activity The next purification step was molecular sieving where we obtained good resolution on a PGM 2000 column eluted by water (while Sephadex gels required dilute buffers with accompanying problems of salt removal) Maximum receptor binding inhibitory activity was obtained in Fraction C with an apparent molecular weight of 1 000–1 200 dalton according to our calibration with peptides and

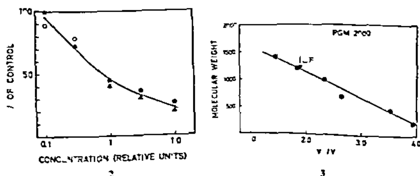


Fig. 2 Log concentration-inhibition curve for Fraction A. The ordinate gives specific = saturable binding of dihydromorphine. The concentration giving 50 inhibition in 0.4 ml buffer was arbitrarily set to 1 unit.

Fig. 3 Molecular weight calibration of the PGM 2000 column. In order of descending molecular weights the following peptides were used Bacitracin, Polymyxin B hexatyrosine, pentaphenylalanine, he aalanine tryptophan. V (= void volume) was determined with serum albumin V is eluted volume The arrow indicates the position of the endogenous factor

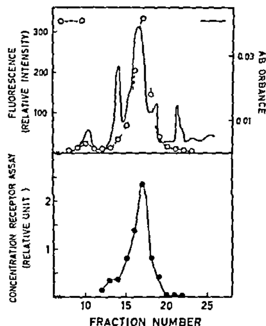


Fig. 4. The upper part gives the elution profile from the PGM 2000 column in terms of fluorescence after reaction with fluorescamine (Fluram, Roche, which reacts with primary amines) and UV absorption at 254 nm. The lower part gives the receptor blockade in relation to elution volume. The units for receptor blockade are those of Fig. 2.

amino acids of known molecular weight (Fig. 3). Fraction B was more complex than Fraction A and was not characterized further. The PGM column effluent from the runs with Fraction A were continuously monitored for UV absorption and also analyzed for primary amines by reaction with fluorescamine. The results (Fig. 4) indicated a coincidence between fluorescamine positive reaction and receptor blocking activity. Some of the UV absorbing material was fluorescamine negative and showed no receptor blocking activity.

The large UV peak which almost coincided with the fluorescamine positive peak had an absorption maximum at 273 nm. It also gave a fluorescence spectrum (excitation maximum 280 nm, emission maximum 320 nm). The fluorescence intensity was highest at neutral pH but very much less at pH 1.5 and somewhat less at pH 11, a typical characteristic for the

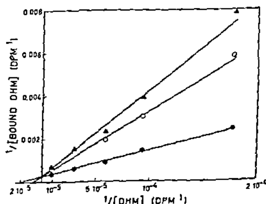


Fig. 5. Double reciprocal analysis of inhibition of dihydromorphine binding to synaptic plasma membrane receptor by the endogenous factor. The lower curve is the control, the upper curves with two concentrations of factor.

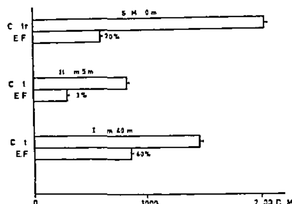


Fig 6 Effect of the endogenous factor (E.F) on dihydromorphine binding to the opiate receptor of synaptic plasma membranes (SPM) and the guinea pig ileum With ileum incubation time was *either 5 or 40 min The inhibition is given* in percent of control (Contr)

aromatic amino acids tryptophan and tyrosine (Shore and Pardee 1956) The receptor blocking activity was not destroyed by boiling at neutral pH for several hours

The nature of the receptor blockade was analyzed in several experiments The binding was reversible and as indicated in Fig 5 the blockade is apparently competitive since in this kind of plot the intersection with the ordinate remained the same in the presence of increasing amounts of the endogenous factor We also found that the opiate receptor of the guinea pig ileum could be blocked by the factor (Fig 6) However the factor was less effective on the ileum than on the SPM preparation particularly after long term incubation

Discussion

The starting point for this investigation was the hypothesis that there might be an endogenous factor with affinity for the opiate receptor There were earlier reports based on bioassays indicating the presence of such factors Thus Green and co-workers (Green *et al* 1963 Robinson *et al* 1967) described a lipoidal material which caused contraction of the isolated guinea pig ileum probably by release of acetylcholine from endogenous stores This effect was completely blocked by very low concentrations of morphine When we followed the extraction scheme of Green and co-workers we could not find any receptor blocking activity Ungar and co workers (Ungar and Cohen 1966 Ungar and Galvan 1968) have described the transfer of morphine tolerance by a low molecular weight compound from a morphine tolerant animal to a non treated animal Very recently Hughes (1975) has found a factor in pig brain which is morphinomimetic on the mouse vas deferens and which is antagonized by naloxone However the complexity of biological assay systems of narcotics makes the interpretation of such experiments in terms of mechanisms of action difficult

The present work shows a different approach to the problem It is based on the hypothesis that the putative endogenous factor might bind to the same receptor site as opiate or affect their binding in an allosteric manner thus allowing its presence to be measured by receptor blockade We know from the work of several groups that the binding area of the opiate receptor is highly discriminative All tested neurotransmitters have affinities which are magnitudes of order lower than those of the narcotics or their antagonists Of a large number of

Acta physiol scand 1975 94 82–94
From the Department of Physiology University of Göteborg, Sweden

Influences on Colonic and Small Intestinal Motility by the Cerebellar Fastigial Nucleus

By

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Received 16 December 1974

Abstract

MARTNER J *Influences on colonic and small intestinal motility by the cerebellar fastigial nucleus* Acta physiol scand 1975 94 82–94

The fastigial influence on intestinal motility was investigated in acute experiments on chloralosed cats Motility was recorded both from the small and large intestine Electrical stimulation of the rostral fastigial pole produced in combination with a blood pressure rise increased motor activity in ileum and colon whil jejunum could respond with either increased or decreased motil ty The intestinal responses were neither secondary to changes in intestinal blood flow nor to baroreceptor reflexes induced by the increased blood pressure The excitatory responses were not due to increased parasympathetic activity since sectioning of such pathways failed to abolish the responses Instead interruption of adrenergic sympathetic discharge accomplished either by guanethidine or by sectioning of relevant nerves did eliminate the responses indicating that the fastigial effects were mediated by suppression of prevailing adrenergic tone Noxious stimuli to the abdomen including laparotomy inhibit intestinal motility by a reflex increase in adrenergic discharge It is suggested that fastigial influence on intestinal motility is mainly due to suppression of this reflex

Besides its well known control of somatomotor function the cerebellum has long been known to exert a widespread influence on autonomically innervated organ systems (*cf* Dow and Moruzzi 1958) Earlier experiments have mainly dealt with the cerebellar cortex while the interest has recently been focused on the cerebellar nuclei especially the fastigial nucleus Thus electrical stimulation of its rostral pole elicits powerful pressor responses (Achari and Downman 1969 Miura and Reis 1969) which have been subject to further analysis in several studies (Achari and Downman 1970 Miura and Reis 1971 Lisander and Martner 1971 1973 Achari Al Ubaidy and Downman 1973) Recently it has been suggested that the fastigial pressor neurons are involved in the control of circulatory orthostatic reflexes (Doba and Reis 1972 1974) but whether they are engaged in the control also of other autonomic functions is not known

While thus information is accumulating regarding cerebellar cardiovascular control much less is known of cerebellar influences on gastrointestinal motility Inhibition of intestinal motility following stimulation of the vermis and the hemispheres has been reported (Voronin and Simkina 1938) and both inhibitory and excitatory gastrointestinal responses

have been observed on stimulation of various cerebellar parts including the fastigial nucleus (Beller and Talan 1971 Manchanda Tandon and Aneja 1972) Beller and Talan (1971) also claimed that rostral fastigial parts were effective in producing excitatory re sponses while caudal parts often produced inhibitory responses

Towards this general background it was considered of interest to investigate in more detail whether the fastigial pressor area besides its influence on cardiovascular control affects also other autonomically innervated organs where the present study was concen trated on the fastigial influence on spontaneous intestinal motility A preliminary report has been published (Lisander and Martner 1974)

Methods

Experiments were performed on 50 cats of either sex which after induction with ether were anesthetized with chloralose 50–60 mg/kg. Free airways were secured by a tracheal cannula

Operat e p oc d s and r cord gs

Arteri l pressure was recorded through a femoral catheter connected to a Statham P 73 AC transducer writing on a Grass polygraph while heart rate was measured by a Grass Tachograph unit. The secret on from the adrenals was eliminated in most animals by encircling l gatures and ad enocorti al substitution was given by i v injection of hydrocortisone (Solu-Gluc® Erco) 10 mg/kg Intestinal motility was recorded in isolated 5 cm long small intestinal and colon c sections gently rinsed from their contents For colonic isolation only loose ligatures were applied to avoid damage to the intramural nervous connections Those parts of the small intestine that were not used for recording were extirpated together with the spleen and the greater omentum After filling the intestinal loops with warm Tyrode solut on motil ty was re corded as intraluminal volume changes transferred via a wide tube to a container of 5 cm diameter which was mounted on a Grass force-displacement transducer FTO3 for continuous recording of its weight The level of the container was adjusted to obtain a constant intraluminal pressure of 5–10 cm H O In some cases small intestinal motility was instead recorded as pressure changes in intralum nal balloons

The vagal nerves were d ssected free in the neck and placed on ligatures so that they could be cut during the experiment The sympathetic supply to the intestines along the superior mesenteric artery could be interrupted by cutting these nerves and also the lumbar colonic ner es were dissected free along the inferior mesenteric artery for subsequent cutting or for revers ble blockade by cooling Also the pelvic nerves were dissected free and placed on ligatures so that they could be cut in the course of the experiment Intestino-intest nal inhibitory reflexes ere elicited either by graded distention of a small isolated intestinal loop or by afferent stimulation of thin mesenteric pedicles

Intestinal blood flow was recorded after heparinization as the outflow from the portal vein after l gation of all venous branches except those from the intestinal segment under study After passing a closed opt cal drop recorder operating an ordinate writer the blood was returned ia the femoral ve n For baroreceptor stimulat on one carotid sinus reg on was isolated and perfused at various pulsating pressures with blood from one of the femoral arteries via a s gmamotor pump The co tralateral sinus nerve was then cut as were the vagal nerves, thus eliminating other baroreceptor st tions

Ce ebell r m lat ons

Topical cerebellar stimulations were induced by means of the Horsley Clarke techn que using a sharp (t p about 50–100 μm in diameter) monopolar stainless steel elect ode insulated except for the tip Square wave pulses of 1 ms duration were delivered by a constant current stimulator at intensities betw n 0 05 and 0 mA corresponding to a voltage ra ge of 1 3 V and with freque cies between 10 and 0 Hz The electrodes were inserted perpendicularly to the stereotaxic horizontal plane after treph nat on and part al removal of the tentorium

Hist l gical pro ed es

After each e periment a small les on w s m de around the elect ode tip by d re t anodal current of 1 mA for 10 s The head of the animal was perfused by sal e followed by a 10 solut o of form ldehyde The

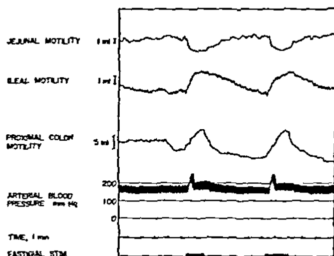


Fig 1 Cat 4.0 kg, Flaxedil 4 mg/kg i v artificial respiration Fastigial stimulation (50 Hz, 1 ms 0 1 mA) produces a marked pressor response while ileal and colonic motility is increased and jejunal motility inhibited

cerebellum and adjacent brainstem structures were then paraffin embedded sectioned in slices of 15 μm and stained by the Nissl technique or in some cases by cresyl fast violet (Kluver-Barrera) In later experiments the cerebellum was frozen sectioned

Drugs

Respiratory interferences with the motility recordings were sometimes impossible to avoid in which cases Gallamine iodide (Flaxidil®) 4 mg/kg b wt. was given artificial respiration was then maintained by a respiratory pump Pharmacological adrenergic blockade was induced by guanethidine (Ismelin® CIBA) 4 mg/kg b wt. i v For α blockade dibenzyline (Smith Kline and French) 2–10 mg/kg b wt i v was used β blockade was induced by propranolol (Inderal® ICI) 0 5–1 mg kg b wt i v Atropine (atropine sulphate) was used in a dose of 0 5–1 mg/kg b wt. i v

Results

Electrical stimulation of the fastigial nucleus affected small intestinal and colonic motility in almost all experiments The response patterns most commonly found when all nervous influences were still intact are shown in Fig. 1 where fastigial stimulation elicits increased colonic and ileal motility while jejunal motility is inhibited. Sometimes however fastigial stimulation produced excitatory jejunal responses as well

1 *Fastigial influence on jejunal motility* While the colon and ileum uniformly responded with increased motility upon fastigial stimulation the jejunum exhibited inhibitory responses in about half the experiments (11 cats) and excitatory responses in the other half (12 cats) In some cats both response patterns could be seen e g initial inhibitions which changed into excitatory responses later on, such a reversal occurring without any change of electrode position During periods of jejunal inactivity increased motility was sometimes elicited as a rebound phenomenon upon cessation of the stimulation The excitatory jejunal responses upon fastigial stimulation were not abolished by vagotomy (6 cats) but by cutting the adrenergic nerve supply or by guanethidine administration (4 cats)

The background of the jejunal inhibitions was more difficult to reveal since these respon

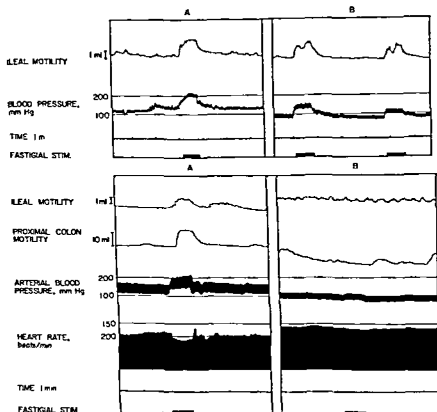


Fig Upper section Cat 2 4 kg. Effect of fastigial stimulat ons (50 Hz, 1 ms 0.. mA) before (panel A) and after (panel B) b lateral vagotomy Note that the prompt ileal excitatory response remains after vagotomy

Lower sect on Cat 3 0 kg fastigial stimulation (50 Hz, 1 ms 0 04 mA) Between panels A and B guanethidine 4 mg/kg b wt i v., has been given. The fastigially induced excitatory intestinal responses in A are abol shed in B while both the colon and ileum now exhibit spontaneous motil ty

ses were not as reproducible as the excitatory ones but they appeared to require int..ct adrenergic mechanisms while they persisted after vagotomy

2 *Fastigial influences on ileal motility* In virtually all cats (39 out of 42) the fastigial pressor response was associated with a prompt increase of ileal motility and inhibitions were never observed The ileal motor responses were often very powerful and direct inspec tions sometimes showed intensely contracted even blanched ileal segments Analysis of the pathways conveying these excitatory ileal responses revealed that the vagal nerves were not necessary (upper section of Fig. 2, A and B before and after vagotomy) Thus cutting the vagal nerves during the experiment in no way eliminated the motility increase to fastigial stimulation though the response was in a few cases somewhat reduced. On the other hand, sectioning of the adrenergic supply eliminated the ileal excitatory responses (3 cats) whether the vagal nerves were cut or not as did administration of guanethidine (7 cats) illustrated in Fig 2 lower section The effect of α-adrenergic blocking agents was then more variable-

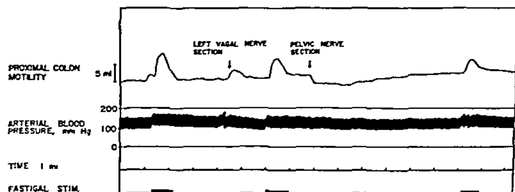


Fig. 3 Cat 2 kg right vagal nerve cut. Fastigial stimulation (50 Hz, 1 ms, 0 2 mA) elicits powerful colonic contraction which persists after complete vagotomy and also after bilateral pelvic nerve section, though somewhat reduced in extent.

in some cats the response vanished but in others it persisted although now appearing first after a delay of some 30 s

3 *Fastigial influence on colonic motility* The *proximal* colon responded in 37 out of 40 cats with contractions upon fastigial pressor stimulation as did *middle* parts of the colon These contractions usually occurred with few seconds delay and persisted throughout the 30–60 s stimulation period to cease promptly when stimulation was discontinued

The distal colon yielded more variable results with no responses in 2 cats and with weak and delayed contractions in three cats These responses were however not due to peristalsis conducted distally from the proximal colon as they could still be obtained when the continuity of the gut was broken Often these colonic responses became gradually less pronounced in the course of the experiment which was hardly due to any cerebellar damage around the electrode since the pressor response never decreased

The mediation of the colonic excitatory responses to fastigial stimulation was analysed either by consecutive sectioning of the peripheral nerves or by using pharmacological blocking agents The effect of sectioning the cholinergic pathways were analysed in 20 cats Fig. 3 illustrates such an experiment Cutting the left vagal nerve (the right one had been earlier cut) slightly reduced but did not abolish the colonic response If also the pelvic nerves were cut, the response was further reduced but not abolished—a persistent finding in all these experiments Cholinergic blockade with atropine (1 mg/kg b wt i v) considerably reduced and delayed the motility increases to fastigial stimulation but did in most cases not abolish them (Fig. 4 panel B) Pelvic nerve sectioning did not even now abolish the response while guanethidine did so (panel C)

The adrenergic pathways are mainly in the lumbar colonic nerves supplying the entire colon, while the proximal colon receives adrenergic innervation also from the thoracic sympathetic outflow via the splanchnic nerves (*cf* Hultén 1969) Interpretation of the results after adrenergic blockade is difficult because this procedure always brings about a marked increase in spontaneous colonic motility which might mask the effect of fastigial stimulation. However after sectioning or cooling the lumbar colonic nerves no clearcut colonic respon

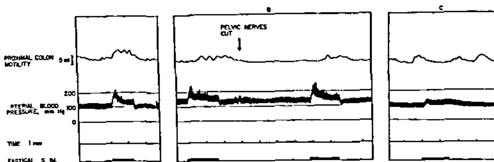


Fig 4 Cat 2.5 kg vagal nerves cut. A Prompt colonic excitation elicited by fastigial stimulation (50 Hz, 1 ms 0 2 mA) B Colonic response after atropine 1 mg/kg between the first and second stimulations the pelvic nerves are cut, which reduces and delays the response C Guanethidine 4 mg/kg, increases *spontaneous colonic motility but eliminates response to fastigial stimulation*

ses could ever be recorded upon fastigial stimulation (9 cats) If the thoracic sympathetic supply was interrupted by cutting the nerves along the superior mesenteric artery the responses were sometimes reduced but could still be obtained The adrenergic blocking drug guanethidine also increased the basal colonic motility but abolished in all 14 cats so treated the earlier very powerful contractions upon fastigial stimulation (Fig 2 lower section)

The α blocking agent dibenzyline (4 mg/kg) rendered the colon more prone to contract spontaneously but sometimes failed to eliminate the colonic responses to fastigial stimulation If dibenzyline was combined with a β adrenergic blocking agent (propranolol 0 5–1 mg/kg b wt i v) the colonic responses were further reduced but not always completely eliminated

4 *Suppression of motility by fastigially induced adrenal catecholamine release* The adrenals were as mentioned excluded in most experiments by ligation to eliminate the effects of circulating catecholamines liberated by fastigial stimulation (Lisander and Martner 1973) If however the adrenal glands were left intact and guanethidine given to eliminate the neurogenic adrenergic influences on intestinal motility fastigial stimulation induced long lasting inhibitory effects in all intestinal segments studied (Fig 5) The delay in onset and the longlasting responses with regard to intestinal motility blood flow and heart rate strongly speaks in favour of a hormonal mechanism It is known that guanethidine does not affect the medullary release of catecholamines but rather enhances their effects (Abercrombie and Davies 1963)

5 *Are the motility responses due to intestinal blood flow changes or to baroreceptor reflexes?* To exclude that the fastigial influence on intestinal motility was secondary to changes in blood flow during stimulation arrangements were made in 4 experiments for simultaneous recordings of intestinal motility and blood flow Although there was a substantial resistance increase in the intestinal vascular bed during fastigial stimulation intestinal blood flow was only slightly affected because of the concomitant pressure rise Independent of the slight changes of blood flow increased motility was constantly elicited by fastigial pressor area stimulation

If a blood pressure rise of same magnitude as that induced by fastigial pressor area

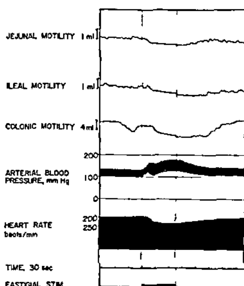


Fig 5 Cat 2 7 kg intact adrenals but guanethidine 4 mg/kg given Note the inhibition of motility in all intestinal parts upon fastigial stimulation The delayed onset and longlasting effects of the cardio vascular and intestinal responses point towards a hormonal mechanism The small regular oscillations seen in the motility recordings reflect increased respiratory movements sometimes obtained by fastigial stimulation

stimulation was caused by either baroreceptor unloading or by rapid dextrane–Tyrode in fusion these procedures would reflexly decrease or increase respectively intestinal blood flow The procedures did not however affect intestinal motility The possibility that en hanced intestinal motility during fastigial stimulation was due to a reflexly reduced adren ergic tone evoked via the baroreceptors upon the fastigial pressor response was excluded by the type of experiment illustrated in Fig 6 When the isolated carotid sinus region was exposed to an increased pressure no change in ileal motility was seen while fastigial stimulation performed during this period of intensified baroreceptor activation still en hanced ileal motility

6 *Fastigial suppression of the intestino intestinal inhibitory reflex* Distention of a gastro-intestinal segment stimulation of afferent mesenteric fibres or noxious abdominal stimuli produce prompt adrenergic inhibition of gastrointestinal motility procedures here included

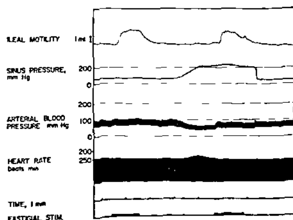


Fig 6 Cat 2 6 kg both vagi and right carotid sinus nerve cut left carotid sinus prepared for pressure perfusion Fastigial stimulation (50 Hz 1 ms 0 2 mA) elicits similar ileal excitatory responses whether the right sinus region is exposed to systemic pressure (left) or to high constant pressure (right) which causes a reflex arterial pressure reduction and brady cardia

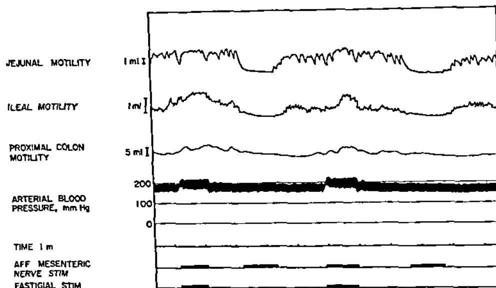


Fig 7 Cat 2 0 kg vagal nerves cut Afferent mesenteric nerve stimulat on (5 Hz, 1 ms 7 V) induces inhibitions of spontaneous intestinal mot lity but this reflex inhibitory response is blocked by concomitant *fastigial stimulation*

in the t rm intestino-intestinal inhibitory reflexes (*cf* Youmans 1968) Fig 7 shows such a reflex intestinal inhibition caused by afferent mesenteric nerve stimulation When a simultaneous fastigial stimulation is performed these reflex inhibitions can often be completely blocked Such fastigial suppressions of intestinal reflex inhibition could be demonstrated both in the small intestine and in the colon although the colonic effects were more variable and could not always be completely suppressed

7 *Histological examination of the fastigial points stimulated* The fastigial pressor area is located in ventro-medial parts of the fastigial rostral pole (Miura and Reis 1969) The fastigial parts affecting the intestinal tract correspond quite well with those producing pressor responses as illustrated in Fig 8 Thus virtually all stimulation points yielding intestinal responses also caused pressor responses and were gathered in ventro-medial parts of the fastigial rostral pole and in the adjacent ventro-rostral cerebellar white matter Effective stimulation points were plotted on maps schematically drawn from frontal sections corresponding to the coordinates P 8 0–P 9 0 given by Snider and Niemer (1961) In Fig 8 the small intestinal responses are shown on the left side of each drawing while the colonic ones are shown on the right side Virtually all responsive points were combined with generally marked pressor responses The likelihood of inducing intestinal responses increased with the intensity of the pressor responses although a marked pressor response was not throughout a prerequisite as can be seen in Fig 3 and 6 where only small to moderate blood pressure increases are recorded The points in Fig 8 representing effective motility responses in the various *intestinal segments seem to be intermingled and localized* in or close to the ventro-medial border of the rostral fastigial parts

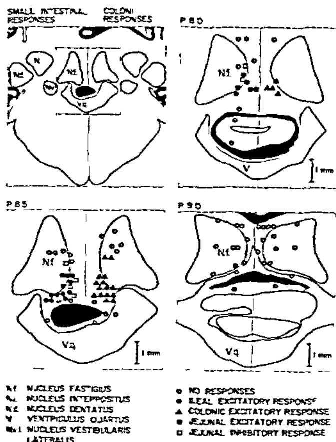


Fig. 8 Frontal section through cerebellum and brainstem. The area within the hatched lines seen in the upper left drawing is magnified and in the subsequent drawings shown at three different levels 8 8.5 and 9 mm posterior to the interaural line. The colonic responses are marked on the right side while the small intestinal responses are shown on the left side of each figure. Explanation of signs and abbreviations are shown in the figure. For further information see text.

Discussion

The present study shows that fastigial stimulation can influence the "spontaneous" motility in the jejunum, ileum and colon. The responses displayed regional differences, with uniformly excitatory responses in the colon and the ileum in the prevailing experimental situations, while the jejunum could show both inhibition and excitation. The neurogenic excitatory colonic and ileal responses, elicited by fastigial stimulation, can theoretically be achieved by an increased parasympathetic activity or/and by a reduced sympathetic tone. Here the situation for the colon is particularly complex as it receives parasympathetic cholinergic innervation from the vagal nerves, which convey excitatory impulses restricted to proximal parts, while the sacral parasympathetic outflow supplies the entire colon via the pelvic nerves. Likewise, the adrenergic innervation is divided into a rostral division, supplying the proximal colon via the splanchnic nerves while the lumbar colonic nerves innervate largely the entire colon (*cf* Hulten 1969 Rostad 1973)

In general, sectioning of the cholinergic supply could be performed without abolishing the colonic and ileal excitatory responses, indicating that they were conveyed by a suppression of an adrenergic influence. Accordingly cutting the mentioned sympathetic nerves, or administration of guanethidine, abolished the excitatory responses to fastigial stimulation.

The fact that atropine markedly reduced and occasionally abolished these excitatory responses by no means speaks against the involvement of primarily adrenergic mechanisms The reason is that adrenergic inhibitory influence on gastrointestinal motility is generally exerted at the intramural *ganglionic* level and thus calls for a prevailing activity in the intramural excitatory cholinergic neurons to be revealed (Norberg 1964 Kewenter 1965 Jansson and Martinsson 1966 Hulten 1969) Atropine will eliminate their excitatory influence on the intestinal smooth muscles but not necessarily a sectioning of the preganglionic cholinergic fibres as long as an intrinsic activity is maintained in the intramural cholinergic plexa Therefore in the latter case a central suppression of a prevailing adrenergic inhibitory influence will reveal itself as an excitatory influence on intestinal motility Thus the cerebellum in general exerts its main effects on spontaneous intestinal motility by decreasing adrenergic activity via the fastigial nuclei while the cerebellar cortex in turn is known to exert a generally inhibitory influence on fastigial activity

It is generally assumed that the adrenergic inhibitory influence at the ganglionic level is exerted by α receptor mechanisms (*cf* Furness and Costa 1974) but even large doses of dibenzyline or/and phentolamine did not always abolish the fastigially induced intestinal contractions although they were now more delayed in onset at the same time as the intestine exhibited a markedly increased spontaneous motility This remaining excitatory response after complete nerve blockade could still be detected after cutting the pelvic and vagal nerves However some cholinergic excitatory fibres appear to reach the colon via its sympathetic supply (Varagic 1956 Hultén 1969) and possibly such cholinergic fibres may have been activated by fastigial stimulation in analogy to the finding that the fastigial nucleus can modify *e g* the cardiac vagal nerve activity (Lisander and Martner 1971 Achari *et al* 1973) Whatever the case the suppression of the adrenergic fibre activity to the gastrointestinal tract appears to be the most prominent excitatory influence on gastrointestinal activity exerted by the fastigial neurons

There seems to be a good correlation between the fastigial pressor response and the fastigial influence on intestinal motility powerful pressor responses being regularly associated with pronounced intestinal excitatory responses and *vice versa* As shown in Fig 8 the effective stimulation points are clustered around the rostral ventro-medial fastigial pole and just ventromedially to the nucleus presumably then representing the efferent pathways from the nucleus The majority of the neurons in rostral fastigial parts send their axons ventromedially and as crossed fibres giving rise to the hook bundle (Matsushita and Iwahori 1971)

No clearcut topographical fastigial organisation could be traced concerning responses in different gastrointestinal parts instead effective stimulation points usually induced responses throughout the intestinal tract However it should be realized that the current spread might amount to 0 5 mm (Wise 1972) and that locating the electrode may have an error of up to 0 5 mm due to the size of the lesion induced

The close association between cardiovascular pressor responses and intestinal responses to fastigial stimulation might imply that the latter were a consequence of the former being for example secondary to neurogenic changes in intestinal blood flow Thus mechanical reductions of intestinal blood flow can inhibit intestinal motility though first when the

reduction in flow is quite marked (Celander 1959 Kock 1959) To exclude any such interactions intestinal blood flow and motility were recorded simultaneously in some experiments revealing that the changes in blood flow secondary to fastigial stimulation were quite small. It is therefore unlikely that *e g.*, the jejunal inhibitory responses to fastigial stimulation should be secondary to any reduced jejunal blood flow Furthermore in the colon and ileum the motility responses were excitatory making it still more unlikely that they were secondary to changes in blood flow Finally the motility responses were usually prompt in onset, making it highly unlikely that they should be secondary to the gradual change in local chemical environment induced by the neurogenic restriction in blood supply

Intestinal motility may be influenced by adrenal catecholamine release induced by fastigial stimulation. When the adrenals were left intact and guanethidine was given to block the neurogenic intestinal responses delayed but longlasting motility inhibitions were obtained as a result of the catecholamine release This illustrates the importance of eliminating the adrenals in studies of neurogenically mediated influences from the cerebellum on gastrointestinal motility In the vast majority of the present experiments the adrenal glands were however excluded in which situation the jejunal inhibition must be ascribed to nervous mechanisms.

Finally the possibility remains that the intestinal responses were secondary to baroreceptor reflex influences induced by the fastigial pressor responses Such reflexes might, for example suppress the sympathetic discharge to the intestine However such a mode of influence was excluded by the fact that even intense and constant baroreceptor activations did not influence the changes in intestinal motility elicited by fastigial stimulation

It is highly unlikely that the cerebellum has any direct nervous connections with the ly controlled organ systems and its influence is probably rather one of modifying the reflex arcs controlling visceral functions Consequently the fastigial responses would be dependent on a prevailing activity in such arcs which, in turn is likely to vary considerably with the experimental situation This may explain the varying jejunal responses to fastigial stimulation sometimes changing from inhibition to excitation in the course of the same experiment The dependence on the experimental situation has a bearing also on comparisons of results in different studies of cerebellar autonomic function In the present study the experimental procedures certainly tend to elicit the spinally mediated *intestino-intestinal inhibitory reflex by means of the inevitable abdominal surgery* inducing sympathetic discharge to the entire gastrointestinal tract As discussed above the fastigial nucleus appears to exert its main control of intestinal motility by modulating adrenergic tone where the preferential influence in the present situation is one of suppression. The spinal intestino-intestinal reflex is subject to control from higher CNS levels and can for example be efficiently suppressed from the medullary "depressor area" (Johansson Jonsson and Ljung 1965 1968)

Besides by operative trauma, the intestino-intestinal inhibitory reflex can be elicited also by electrical stimulation of mesenteric afferents or by intestinal distension procedures which can be graded and, if strong enough are able to entirely inhibit intestinal motility In case the fastigial nucleus is effective in suppressing the intestino-intestinal reflex, fastigial stimulation would be expected to block the reflex inhibition induced by *e g* afferent mesen

teric stimulation which was indeed the case (Fig 7) If however supramaximal afferent stimulation was applied for eliciting the reflex it was increasingly difficult to block it by fastigial stimulation

A regional difference in sympathetic control of the small intestine has been described by Kewenter (1965) with a preponderant inhibitory influence on the ileum This may explain why the jejunal and ileal responses to fastigial stimulation were often different As fastigial stimulation seems to uniformly suppress sympathetic activity to the gastrointestinal tract it would be expected that the resulting increases in motility would be particularly pronounced in those sections of the small intestine where the adrenergic inhibitory influence was strongest to start with Such a view corresponds well with the response pattern generally found in the small intestine

In conclusion the fastigial nucleus and hence the cerebellum is involved in the autonomic regulation of intestinal motility where one important mode of action appears to be a modulation of adrenergic inhibitory discharge A possible mechanism for this might be fastigial suppression of the spinal intestino-intestinal inhibitory reflex which is conveyed via adrenergic fibre suppression of the activity in the intramural cholinergic ganglion cells Indeed results from recent experiments to be reported in a subsequent paper strongly support such a mechanism This by no means denies the possibility that cerebellar control of intestinal autonomic regulation may be exerted also by other mechanisms that might be revealed during other experimental conditions

This research has been sponsored by grants from the Swedish Med cal Research Council (No B74 14X 16 10C) from Svenska Sällskapet for Medicinsk Forskning and from the Faculty of Medicine Univers ty of Göteborg.

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Acta physiol scand 1975 94 95–104
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Influences on the Defecation and Micturition Reflexes by the Cerebellar Fastigial Nucleus

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Received 16 December 1974

Abstract

MARTNER J *Influences of the defecation and micturition reflexes by the cerebellar fastigial nucleus* Acta physiol scand *1975 94 95–104*

The influence of the cerebellar fastigial nucleus on the defecation and micturition reflexes was investigated in chloralosed cats with recordings of colonic blood flow and motility intravesical and intra abdominal pressures Whenever effective topical fastigial stimulation regularly suppressed both somatomotor and autonomic components of the defecation reflex to the extent that the straining movements as well as the colonic vasodilatator and motor responses associated with defecation could be completely inhibited Bladder motility could either be suppressed or enhanced depending both on prevailing bladder tone and on the fastigial site stimulated The autonomically conveyed inhibitory responses were in both cases independent of the adrenergic sympathetic pathways since they were unaffected both by sympathetic nerve sectioning and by adrenergic blocking drugs but eliminated by pelvic nerve section It is suggested that the mentioned fastigial inhibitory influences are exerted on the spinal parasympathetic reflexes controlling the bladder and colon Parallels between fastigial control of autonomic and somatomotor mechanisms are discussed

Increasing evidence is accumulating in support of a role for the cerebellum also in autonomic control Especially interesting in this context is the fastigial nucleus which is very potent in eliciting both cardiovascular (Achari and Downman 1969 Miura and Reis 1969) and gastrointestinal responses (Lisander and Martner 1974) upon electrical stimulation Both types of responses are induced from the rostral ventromedial fastigial pole and involve predominantly a modulation of adrenergic sympathetic discharge which is enhanced to the circulatory system but as has been recently shown is generally suppressed to the colon and the small intestine (Martner 1975) It was considered of interest to investigate whether also parasympathetically conveyed autonomic responses can be directly influenced from the fastigial nucleus where especially the defecation and micturition reflexes are of relevance To fully reveal the fastigial influence on the parasympathetically induced colonic motor responses during defecation it was necessary to eliminate direct fastigial adrenergic modulation of the colon (Martner 1975)

A possible fastigial influence on the defecation reflex is of particular interest also from another point of view insofar as it involves easily elicited somatomotor and autonomic components The former requires for its full expression supraspinal levels while the la

is basically conveyed at spinal sacral levels (Garry 1933) though being considerably influenced also by bulbar and suprabulbar autonomic centres (Hatcher and Weiss 1923 Koppanyi 1930 Hess and Brugger 1943)

While the cerebellar control of somatomotor mechanisms is by now fairly well understood comparatively little is known about the principles of cerebellar autonomic control Therefore investigations of cerebellar effects on reflexes that involve both somatomotor and autonomic components might help to elucidate how the cerebellum influences autonomic mechanisms For such reasons the first part of the present study deals with the fastigial influences of the somatomotor and autonomic components of the defecation reflex

Concerning the urinary bladder several authors have reported cerebellar excitatory as well as inhibitory influences especially from the vermal cortex (Whiteside and Guyton 1952 Emerson *et al* 1961 Bruhn *et al* 1961 Rasheed Manchanda and Anand 1970) Also from the fastigial nucleus both micturition (Chambers 1947) and suppression of the micturition reflex (Bradley and Teague 1969) can be elicited and bladder tone can be either increased or decreased (Bellar and Talan 1971 Manchanda and Bhattarai 1972 Lisander and Martner 1974) To further characterize the fastigial structures influencing autonomic mechanisms it was considered of interest to investigate whether they influence also bladder motility in a more regular and specific way

A brief preliminary report covering part of the present results has earlier been presented (Lisander and Martner 1974)

Methods

Experiments were performed on 30 cats anesthetized by chloralose 50–60 mg/kg b wt Free airways were secured by a tracheal cannula

Stimulations For cerebellar stimulation the Horsley Clarke technique was utilized exploring the fastigial nucleus by electrodes inserted perpendicularly to the horizontal plane Stimulation was performed via thin monopolar stainless steel electrodes insulated except for the tip (about 50–100 μm in diameter) using square wave pulses of 1 ms from a constant current stimulator The intensities used were varied between 0 03 and 0 3 mA (corresponding to a voltage range from 1 to 3 5 V) at a frequency range of 10 to 50 Hz After each experiment a small lesion was induced around the electrode tip by an anodal current of 1 mA for 10 s The head of the animal was then perfused by saline followed by a 10 solution of formaldehyde relevant cerebellar parts were frozen sectioned and the stimulation points identified

Recordings Arterial pressure was recorded through a femoral catheter connected to a Statham P 23 AC transducer writing on a Grass polygraph Heart rate was measured by connecting the pressure recording amplifier to a Grass Tachograph unit When bladder pressure was recorded the abdominal wall overlying the urethra was opened by a small incision and a catheter inserted into the bladder via the urethra Variations in intravesical pressure reflecting bladder motility were then recorded by connecting the catheter to a transducer A saline reservoir adjustable to various levels was connected to the catheter via a side branch thus allowing for bladder filling at different pressures Sometimes the abdominal incision was extended so that the hypogastric and pelvic nerves could be isolated placed on ligatures and cut in the course of the experiment

The defecation reflex was elicited either by afferent stimulation of one cut pelvic nerve the other being left intact or by applying a mechanical frictional stimulation to the rectal mucosa (*cf* Hultén 1969) For recording colonic motility and blood flow the abdominal wall was opened by a midline incision and the small intestine was extirpated together with the spleen and greater omentum About 5 cm of the most proximal colonic loop was isolated gently rinsed from contents and filled with Tyrode solution The loop was distally isolated by loose ligatures to avoid damage of the intramural nervous connections Colonic motility was recorded as intraluminal volume changes transmitted via a wide tube to a container with a diameter of 5 cm mounted on a Grass force displacement transducer FTO 3 thus continuously recording the weight of the container The level of the container was adjusted to obtain an intraluminal

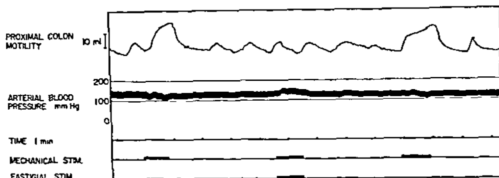


Fig 1 Cat 3 0 kg. Adrenergic fibres blocked by guaneth dine 4 mg/kg. Mechanical rectal stimulation elicits powerful colonic contractions superimposed on spontaneous motility Note that a concomitant fastigial stimulation (50 Hz, 1 ms 0 04 mA) completely abolishes the colonic motor responses

pressure of 5–10 cm H_2O Colonic blood flow was determined in heparinized animals by recording the venous outflow from the superior mesenteric ve n after extirpation of the small intestine After passing a closed opt cal drop recorde operating an ordinate writer the blood was returned to the animal via the femoral vein

D ugs Since respiratory mo ements sometimes interfered with the colonic events gallamine iodide (Flaxedil®) 4 mg/kg b wt i v was in these cases gi en to avoid such disturbances, whereupon artificial resp rat on was gi en No muscle paralys ng drugs were however gi en when the straining movements in connection with defecation were to be recorded these were registered as pressure increases in an intraabdominal balloon In most exper ments the secretion from the adrenals was eliminated by encircling l gatures around both glands and adrenocortical substitution was then given by i v injection of hydrocortisone (Solu-Gluc® Erco) 10 mg kg b wt. In some experiments the lumbar colonic nerves were dis sected free from the inferior mesenteric artery and cut distally to the gangl on but in most cases the adrenergic supply to the colon was instead blocked by guanethidine (Ismelin® CIBA) 4 mg kg b wt i v sometimes in combination with d benzyline (Smith Kline and French) 4 mg/kg b wt. i v As an α blocking agent phentolam ne (Regitin® CIBA 1 mg kg b wt. i v) was also used β-blockade was induced by propranolol (Inderal® ICI) 0 5–1 mg kg b wt i v Atrop ne (atropine sulphate) was used in a dose of 0 5-1 mg kg b wt i v

Results

1 *Fastigial influence on defecation*

Defecation involves activation of both somatomotor and autonomic reflex arcs where straining movements increase the intraabdominal pressure and together with colonic contractions help to expel the faeces Colonic vasodilatation also forms part of the pattern (Hultén 1969) All these reflexly induced changes were found to be influenced by fastigial stimu lation As illustrated in Fig 1 the reflex colonic contractions elicited by mechanical rectal stimulation could be completely suppressed in the adrenergically blocked animals This was the case in 7 cats and only in 2 expts fastigial stimulation failed to attenuate the colonic response Likewise reflex colonic contractions induced by afferent pelvic nerve stimulation were inhibited as well (2 cats) Atropine reduced but did not eliminate the reflex colonic contractions and the small remaining reflex motor responses to rectal stimulation were also suppressed by fastigial stimulation

Fig 2 shows that the straining movements reflexly induced by afferent pelvic nerve stimulation could be completely suppressed by fastigial stimulations Mechanical rectal

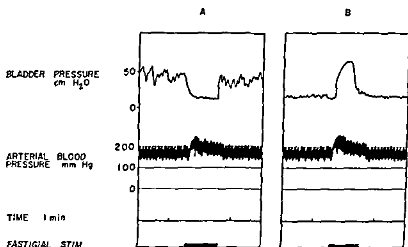


Fig. 5 Cat 2 8 kg Fastigial stimulation (50 Hz 1 ms 0 1 mA) induces inhibition of bladder motility when performed against a background of high sustained intravesical pressure (panel A) The same fastigial site elicits bladder contraction if stimulation is performed during a background of low bladder tone The small oscillations in bladder pressure seen during bladder inhibition are due to respiratory movements

fastigial influence could now be traced Atropine (1 mg/kg b wt) substantially reduced the reflex bladder contractions but fastigial stimulation suppressed also the small remaining contraction waves

Bladder contractions could also result upon fastigial stimulation although they were not so easily demonstrated as were the bladder inhibitions However once appearing they could be quite prominent (Fig 5 panel B) This figure further illustrates that the very same fastigial point from which excitatory responses could be elicited (panel B) could also depress bladder motility once a pronounced and sustained reflex bladder tone was at hand (panel A) Neither the excitatory nor the inhibitory bladder response were strictly confined to the fastigial pressor area although the former type of response was usually associated with the pressor response

The left side of Fig 6 summarizes the points from which bladder responses were elicited The fastigial nucleus can evidently not be divided into one excitatory and one inhibitory area in this very respect even though the bladder inhibitory responses (open triangles) tend to cover a larger area Contrary to most other fastigial autonomic effects including bladder excitation the inhibitory bladder response seems to follow an all or nothing law Thus while low stimulation frequencies did not noticeably influence bladder tone a gradual frequency increase suddenly induced total suppression of bladder contraction usually when 10-20 Hz were reached This frequency range corresponds well with that where fastigial influences on *e g* blood pressure and gastrointestinal motility become obvious (Lisander and Martner 1975) However the latter type of responses essentially dependent of discharge changes in the sympathetic adrenergic fibres are characterized by a gradually rising frequency-response curve which reaches a plateau at 40-50 Hz while the para sympathetically mediated bladder inhibitory responses once appearing were then always maximal *i e* producing 100 per cent suppression of the bladder motility

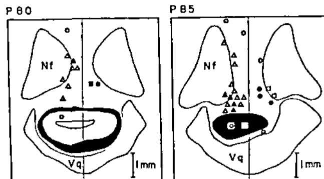


Fig 6 Cerebellar frontal sections 8 and 8 5 mm posterior to the interaural line The bladder responses are shown to the left and the defecation responses to the right Open circles No response Open triangles Inhibitory bladder responses Filled triangles Excitatory bladder responses Filled dots Suppression of colonic motor responses Filled squares Suppression of colonic vasodilatation Open squares Suppression of straining movements Nf Nucleus fastigius Vq Ventriculus quartus

Discussion

Colonic motility elicited from the rectum includes profound reflex contractions particularly of the proximal but also of the distal colon (Schmidt and Fulgraff 1963) where both afferent and efferent pathways run in the pelvic nerves (Hulten 1969) The defecation reflex can be elicited either by mechanical rectal stimulation or by pelvic afferent stimulation In both cases fastigial stimulation could completely abolish the colonic motor response showing that the fastigial influence cannot be a matter of *e g* any neurogenically conveyed suppression of receptor sensitivity in the rectal mucosa Hypothalamic stimulation can inhibit spontaneous colonic motility (Wang *et al* 1940 Sheehan 1942 Rostad 1973) and the latter investigator found that this inhibitory effect was abolished by adrenergic blockade In contrast the fastigially induced suppressions of reflex colonic motility reported here were due to a direct inhibition of the parasympathetically mediated defection reflex and were not a matter of any activation of colonic sympathetic fibres obvious from the fact that all animals used were subject either to pharmacological adrenergic fibre blockade or to section of the lumbar colonic nerves This procedure was also necessary for the full expression of the parasympathetically mediated reflex colonic motor and vasodilator responses which are known to be suppressed by a prevailing sympathetic discharge presumably at the intramural ganglionic level (Hultén 1969) Moreover the fastigial influence on spontaneous colonic motility in cats is excitatory in nature and mediated by a suppression of prevailing sympathetic discharge (Lisander and Martner 1974) Elimination of the sympathetic inhibitory influence on parasympathetically mediated colonic responses which would certainly interfere with the present type of study was another reason for using various kinds of adrenergic blockade Ligation of the adrenals excluded the possibility that the fastigial inhibition of the reflex colonic motility was mediated by a release of catecholamines from the adrenal medulla (Lisander and Martner 1973)

Besides influencing colonic motor activity fastigial stimulation could also completely suppress the reflex vasodilatation accompanying defecation Efferent pelvic nerve stimulation elicits mucosal vasodilatation and secretion (Wright Florey and Jenning 1938) and Hultén (1969) showed that the vasodilatation forms part of the defecation reflex and is neither eliminated by adrenergic nor by cholinergic blocking agents Whichever the trans

mittor mechanism involved at these parasympathetic nerve endings the fastigial nucleus could completely suppress this vascular response

Interestingly the somatomotor component of the defecation reflex was affected by the same fastigial area and in the same inhibitory way as the autonomic components Thus total suppression of the straining movements was always induced by fastigial stimulation Cerebellar modulations of somatomotor mechanisms are wellknown ever since Lowenthal and Horsley (1897) reported inhibition of decerebrate rigidity by cerebellar stimulation Since the fastigial nucleus always modified the somatomotor and autonomic components of the defecation reflex in the same direction it seems likely that cerebellar control may be exerted according to similar principles both concerning somatomotor and autonomic mechanisms

In fact other parallels between the cerebellar influence on somatomotor and autonomic mechanisms do exist and can be illustrated *e g* by comparing wellknown cerebellar somatomotor effects with the results obtained on bladder autonomic functions It was found in the present experiments that the fastigial nucleus could both inhibit and augment bladder motility in consonance with the findings of Beller and Talan (1971) and Manchanda and Bhattarai (1972) Determinant of the direction of the response was both the prevailing bladder tone and the cerebellar site stimulated Thus moving the electrode only 0 5 mm sometimes changed an inhibitory response into an excitatory one Such a reversal could be obtained even from the same fastigial site inhibitions occurring during periods of reflexly induced bladder contractions but changing into excitatory responses when the contractions had spontaneously subsided Similar effects have been induced from the cerebellar cortex (Whiteside and Guyton 1952) The same kind of variable cerebellar influence de on background activity has been illustrated also concerning the cardiovascular system (Moruzzi 1938) and is also demonstrated concerning somatomotor reflexes (Moruzzi 1936) Thus cerebellar stimulation produced inhibition of extensor hypertonia in decerebrate cats while increased extensor tone was occasionally recorded if stimulation was undertaken *during hypotonia sometimes as a rebound effect at the end of the stimulation* Such rebound phenomena have been observed both for the autonomic and somatomotor components of the defence reaction during sham rage (Zanchetti and Zoccolini 1954) and were *also observed concerning the purely autonomic responses of the bladder in the present* study

The finding that cerebellar stimulation produced a constant and generalized inhibition of *e g* the defecation reflex in the present experiments does however not necessarily imply that the cerebellum normally always exerts a suppressive influence in these respects Electrical stimulation of specific brain areas does not necessarily simulate in vivo patterns in a precise way For example such stimulations excite certainly also antidromically and without discrimination both afferent and efferent pathways and may affect both fibres and neuron somata Hence if effects can be demonstrated in the form of fastigial suppression of defecation and micturition as in this case it no doubt indicates that the cerebellum is involved in the control of these reflexes but reveals little about the way by which it is normally brought about whether concerning levels of influence intraspinal pathways *etc* Thus if topical electrical stimulations are applied somewhere in the complex cerebellar

machinery stereotype response patterns are likely to be obtained *e g* in the form of over all inhibitions while the normal cerebellar coordination might well involve both inhibitory and facilitatory modulations depending on the prevailing input of information It is known that the cerebellum receives afferent projections also from visceral organs (Widén 1955)

From the present data alone it is therefore not possible to decide on what level the cere bellum expresses its autonomic influence It is however most unlikely that there are spe cific cerebellar projections to the effector organs studied rather the results strongly indicate that the cerebellum modifies the spinal autonomic reflex arcs involved at the bulbar or/and spinal levels As for the micturition reflex the cerebellar interaction might take place in the bulbar reticular formation from which descending pathways influence the sacrospinal micturition center A suppression of the parasympathetic micturition reflex following fas tigial stimulation might however *a priori* also be due to sympathetic activation to the bladder perhaps then occurring as a parallel to the intensified sympathetic drive on the circulatory system that constitutes the fastigial pressor response The bladder is *via* the hypogastric nerve supplied with sympathetic fibres that can induce bladder relaxation (Elliott 1907) In the present study however neither pharmacological adrenergic fibre blockade nor sympathetic fibre section affected the fastigially induced depression of bladder motility Thus the bladder inhibitions obtained in the present experiments could not be a consequence of any sympathetic activation Instead the cerebellar influence studied here was in all likelihood due to a suppression of the parasympathetically mediated micturition reflex *per se* exerted either at the bulbar or spinal levels as suggested also by Bradley and Teague (1969) Indeed the fastigial nucleus projects to the bulb and as can be seen from the drawing in Fig 6 responses were also obtained from points along the fastigio-bulbar pathways

Although the bladder responses were not exclusively elicited from the fastigial pressor area (Fig 6) which is restricted to the rostral ventromedial fastigial pole this area proved to be effective in producing both excitatory and inhibitory bladder responses as well as a generalized suppression of the defecation reflex These fastigial effects were not at all dependent on the integrity of sympathetic pathways and consequently this particular cerebellar area is by no means only engaged in sympathetic control but rather seems to be involved in all kinds of autonomic mechanisms sympathetic as well as parasympathetic and also affecting combined autonomic–somatic responses like the defecation reflex

This research has been sponsored by grants from the Swed sh Medical Research Council (no B74 14X 16-10C) from Svenska Sällskapet för Medicinsk Forskning and from the Faculty of Medicine Un vers ty of Goteborg

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Acta physiol scand 1975 94 105–111
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Airway Effects of Slow Reacting Substance, Prostaglandin F_2 and Histamine in the Guinea-Pig

By

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Received 17 December 1974

Abstract

STRANDBERG K and P HEDQVIST *Airway effects of slow reacting substance prostaglandin $F_{2\alpha}$ and histamine in the guinea pig* Acta physiol scand 1975 94 105–111

SRS PGF_2 and histamine were administered intravenously or as aerosols to artificially ventilated guinea pigs in order to assess their capacity to affect tracheal insufflation pressure measured by means of Konzett Rossler technique Independently of route of administration all three compounds increased tracheal insufflation pressure SRS being the most potent one Bilateral cervical vagotomy did not alter the effect Relative to histamine SRS and $PGF_{2\alpha}$ were considerably more active by aerosol administration than by intravenous injection The aerosols had little or no effect on systemic blood pressure On intravenous injection histamine decreased and SRS and PGF_2 increased arterial blood pressure in a dose-dependent fashion The airway effects of histamine were correlated to those on blood pressure whereas with SRS and $PGF_{2\alpha}$ this was not seen when the blood pressure effects were marked Preadministration of adrenaline or isoprenaline as aerosols antagonized the increase in insufflation pressure but not the effects on blood pressure produced by intravenously injected histamine or $PGF_{2\alpha}$

It is concluded that SRS $PGF_{2\alpha}$ and histamine on intravenous or aerosol administration increase tracheal insufflation pressure in the guinea pig mainly by an act on on airway tone. The data emphasize that SRS is a potent bronchoconstricting agent possibly of pathophysiological significance in guinea pig anaphylaxis

The appearance of a non histamine smooth muscle stimulating principle slow reacting substance (SRS) on anaphylactic reaction in the guinea pig lung was first observed by Kellaway and Trethewie (1940) Subsequently SRS has been demonstrated to be formed on histamine release in a variety of tissues and species including man (Chakravarty *et al* 1959 Chakravarty and Uvnäs 1960 Brocklehurst 1960) The mechanism of its formation seems to be closely related to the histamine release process (Chakravarty and Uvnas 1960 Änggård *et al* 1963 Strandberg 1971 a)

The chemical identity of SRS is as yet unknown However SRS resembles the prostaglandins (PGs) in being a releasable lipid-soluble unsaturated hydroxy acid but it is not inactivated by prostaglandin dehydrogenase (Strandberg and Uvnas 1971) The PGs together with histamine and SRS have recently been identified in perfusates from lung tissue undergoing anaphylaxis (Piper and Vane 1969 Piper and Walker 1973) Mainly due to their

constrictor action on bronchial smooth muscle. SRS, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and histamine have been suggested to serve as chemical mediators of anaphylactic bronchoconstriction (cf. Collier and James 1970).

In the present investigation we have studied the action of SRS on airway tone in the anesthetized guinea pig and compared the effect with that of $PGF_{2\alpha}$ and histamine. The compounds have been administered by intravenous injection or as aerosols and changes in the tracheal insufflation pressure have been recorded. Our data demonstrate that, independent of administration route, SRS was the most effective compound in causing increased insufflation pressure. A preliminary account of part of the results has been presented elsewhere (Strandberg 1973).

Methods and materials

Seventeen male albino guinea-pigs (650–935 g) were used for the study. They were anesthetized with sodium pentobarbitone (60 mg/kg) i.p. During the experiments supplements were given i.v. when needed. The trachea was cannulated and the animals were ventilated by the use of a Palmer constant volume respirator. The frequency was [illegible] strokes/min and the stroke volume (4–6 ml) was set according to the weight of the animals. Changes in the insufflation pressure at a constant airflow were measured with a Statham pressure transducer (P23 BC) connected to a side-arm of the tracheal cannula. When required, the expiratory arm of the tracheal cannula was clamped to facilitate the recovery by forced inflation. A catheter was inserted into the right external jugular vein and heparin (500 IU/kg) was given. Systemic blood pressure was recorded from the right common carotid artery using a Statham pressure transducer (P23 AC). The recordings were made on a Grass model D polygraph. Aerosols were generated in a De Vilbiss nebulizer Model 35A nebulizing [illegible] ml/min of the fluid (5 ml). The nebulizer was connected to the air inlet channel of the ventilator. To minimize condensation of water all air passages were kept the shortest possible and were kept warm by lamps. The aerosol reached the tracheal cannula within 5 s.

SRS was obtained from cat paws perfused with a modified Tyrode solution containing compound 48/80 (1 μg/ml). It was purified by solvent extraction, silicic acid and anion exchange chromatography as previously described (Strandberg and Uvnäs 1971). One unit of SRS has been defined as the threshold dose of SRS for a contractile action on the isolated guinea-pig ileum (Strandberg 1971 a). Prostaglandin $F_{2\alpha}$ was a gift from Dr. J. Pike, Upjohn Co., Kalamazoo, Michigan, U.S.A. Histamine chloride was used but the values presented are in terms of the base.

Results

Intravenous administration

On intravenous injection SRS, $PGF_{2\alpha}$ and histamine produced an increase in tracheal insufflation pressure (Fig. 1). Usually the time lapse to reach the peak effect as well as the recovery period were shorter for histamine than for the other 2 compounds. Fig. 2 gives the dose-response curves for the effects on the insufflation pressure. Assuming that 1 unit of SRS is about 1 ng (Strandberg and Uvnäs 1971) the rank order of potency for the compounds was SRS > histamine > $PGF_{2\alpha}$. The doses of SRS and histamine causing 100 % increase in insufflation pressure were significantly lower than that of $PGF_{2\alpha}$ ($p < 0.05$, $n = 5$ according to Student's t test for paired variates).

SRS and $PGF_{2\alpha}$ produced an initial drop in blood pressure succeeded by a prolonged and usually marked increase (Fig. 1). Histamine caused a more marked fall in blood pressure which was sometimes followed by a moderate increase. Histamine consistently produced dose-dependent changes of both tracheal insufflation pressure and systemic blood pressure. Usually this was also observed with SRS and $PGF_{2\alpha}$. However, in some cases, when the

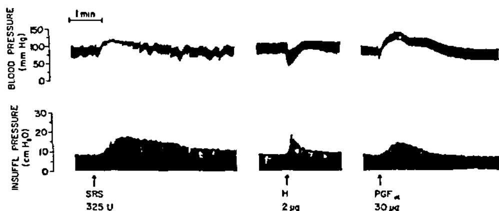


Fig 1 Effects of i v injected SRS (1 unit ≈ 1 ng) PGF $_{2\alpha}$ and histamine on systemic blood pressure and tracheal insufflation pressure in a guinea pig (835 g) anesthetized with pentobarbitone

blood pressure effects were particularly marked and still dose related SRS and PGF$_{2\alpha}$ caused only small and variable changes in insufflation pressure

The increase in insufflation pressure but not the effects on blood pressure produced by intravenously injected histamine or PGF$_{2\alpha}$ was antagonized by a preceding 3 min administration of adrenaline or isoprenaline aerosols Thus after adrenaline (25 µg/ml) the responses to histamine were 27–58 per cent (n = 3) and to PGF$_{2}$ 50–58 per cent (n = 2) of those recorded prior to administration of adrenaline Corresponding figures for isoprenaline (25 µg/ml) (n = 2) were 10–30 per cent and 10–20 per cent respectively

In an attempt to abolish the possible influence of reflex vagal activity on the action of the

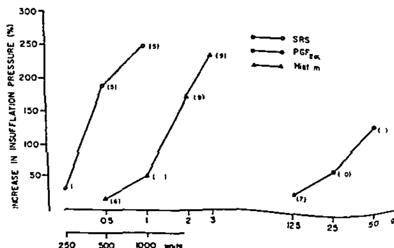


Fig. Dose-response (peak effect) curves for the effects of intravenously administered SRS, PGF and histamine on the tracheal insufflation pressure Mean values are given Within brackets are given the number of observations.

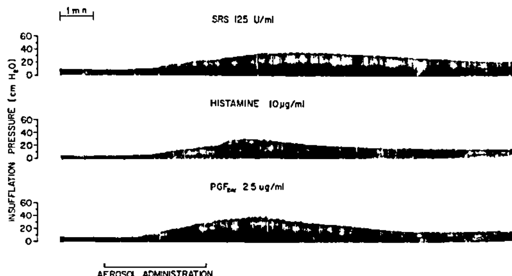


Fig 3 Effects of SRS PGF$_{2\alpha}$ and histamine as aerosols on the tracheal insufflation pressure in a guinea pig (760 g) anesthetized with pentobarbitone Concentrations denote the respective concentrations in the aerosol generator

compounds bilateral cervical vagotomy was performed in 4 expts However this treatment did not significantly decrease either the basal insufflation pressure or the responses to the compounds

Aerosol administration

Fig 3 illustrates the action of SRS PGF$_{2\alpha}$ and histamine administered as aerosols They all produced a dose dependent increase in tracheal insufflation pressure (Fig 4) PGF$_{2\alpha}$ and histamine were largely equiactive but of considerable lower potency than SRS Notably

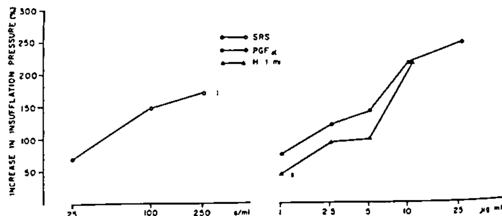


Fig. 4 Dose response (peak effect) curves for the effects of SRS PGF$_2$ and histamine as aerosols on the tracheal insufflation pressure Mean values are given Within brackets are given the number of observations

TABLE I Relative activities of SRS $PGF_{2\alpha}$ and histamine on i v injection and on aerosol administration The histamine dose and concentration respectively increasing tracheal insufflation pressure by 100 is given as 1 0 The activities of SRS and PGF_2 were similarly calculated and are expressed in terms (ratios) of the histamine values The data from Fig. 2 and 4 have been used for the calculations

Compound	Intravenous injection	Aerosol administration
SRS	12	93
PGF_2	0 1	[illegible] 5
Histamine	1 0	1 0

aerosol administration of the three compounds caused no effect or a slight decrease in systemic blood pressure

In Table I the activities of SRS and $PGF_{2\alpha}$ in insufflation pressure are given relative to that of histamine On this basis it seems that both SRS and $PGF_{2\alpha}$ were considerably more active via the aerosol route than given by i v injection An overall comparison of the effects on insufflation pressure will however not be valid since the absolute quantities of the aerosols actually reaching the lower airways could not be determined

Discussion

The aim of this study was to analyze the actions on airway resistance of three potential anaphylactic mediator substances in the guinea pig, *i e* SRS $PGF_{2\alpha}$ and histamine For this purpose the substances were administered either by i v injection or as aerosols and changes in the tracheal insufflation pressure were taken to represent changes in airway resistance It is true that this technique does not necessarily distinguish changes due to pulmonary congestion from those due to changes in bronchial tone (Widdicombe 1963) However due to anatomical considerations the particle size of the aerosols and the almost lack of systemic effects it seems very likely that aerosol administration caused effects predominantly on the airway tone The fact that the increase in insufflation pressure produced by i v injected histamine or PGF_2 was antagonized by aerosols of adrenaline or isoprenaline indicates that also the data obtained on intravenous injection at least in part reflect actions on the airway tone In this context it should be noted that both sympathomimetic compounds relax bronchial muscle but differ conspicuously in their effects on the pulmonary and systemic circulation (*cf* Aviado 1960) In addition there was no true correlation between circulatory effects and effects on insufflation pressure after i v injections of SRS or $PGF_{2\alpha}$ In fact a marked blood pressure increasing effect was commonly associated with small and variable effects on tracheal insufflation pressure Thus there is no compelling reason to assume that circulatory events resulting from i v injection of SRS or $PGF_{2\alpha}$ are of any major importance in the production of increased insufflation pressure

Irrespective of administration route SRS was the most potent of the compounds studied Like PGF the effect of SRS on the tracheal insufflation pressure was more pronounced on aerosol administration than on i v injection when the results were related to the effects of histamine Similar data have been reported for the bronchodilator action of PGF in com

parison to the effect of isoprenaline (Large Leswell and Maxwell 1969) The lung has a high capacity to inactivate PGs independently of whether they are administered directly to lung tissue or given as i v injection (Änggård and Samuelsson 1964 Ferreira and Vane 1967) Concerning SRS both guinea pig serum and lung tissue have been shown to inactivate SRS (Strandberg 1971 b) The relatively higher potency of SRS and $PGF_{2\alpha}$ as aerosols compared to i v injection is therefore probably due to differences in diffusion distance to the target area

On intravenous injection SRS and $PGF_{2\alpha}$ produced an increase in the systemic blood pressure A similar effect of $PGF_{2\alpha}$ is seen in the dog whereas $PGF_{2\alpha}$ has a depressor action in the cat and rabbit (*cf* Karim and Somers 1972) In the dog the pressor effect has been attributed to peripheral venoconstriction leading to an increased venous return and thereby an increase in the cardiac output (Du Charme Weeks and Montgomery 1968) However Nakano and Cole (1969) considered the increased cardiac output a result of the increased myocardial contractility caused by $PGF_{2\alpha}$ The depressor effect of $PGF_{2\alpha}$ and of the other compounds occassionally observed on aerosol administration in the present study may be a circulatory consequence of the prevailing bronchoconstriction

Anaphylactic bronchoconstriction in the guinea pig is considered to be mediated by the release and formation of bronchoconstricting substances *i e* principally histamine SRS $PGF_{2\alpha}$ and kinins in the lung (*cf* Collier and James 1970) Using a variety of chemical antagonists Collier and James (1967) tried to estimate the relative contribution of some of the possible mediators in the anaphylactic reaction They confirmed the generally accepted view that antihistamines render protection and could also show that some non steroidal anti inflammatory compounds e g aspirin and meclofenamate gave additional protection The latter findings were taken to indicate that SRS and kinins contribute to the anaphylactic bronchoconstriction since it was shown that the anti inflammatory compounds used antagonized the actions of partially purified SRS and bradykinin administered i v In the light of recent observations that aspirin indomethacin meclofenamate and allied substances are potent inhibitors of prostaglandin synthetase (Vane 1971 1973) the results could equally well suggest inhibition of the production of PGs during anaphylaxis as also pointed out by Collier (1971) In fact Collier (1971) proposed that SRS and kinins exert their effects in the guinea pig lung via activation of PG biosynthesis However peroral pretreatment of sensitized guinea pig with high doses of indomethacin does not protect the animals from developing respiratory distress and convulsions on inhalation of antigen although the production of PGF is inhibited (Strandberg and Hamberg 1974) This seems to indicate that at least $PGF_{2\alpha}$ is no major mediator in anaphylactic bronchoconstriction in the guinea pig It is even questionable if histamine is of prime importance for this reaction since it has been demonstrated that the protective effect of the anti histaminic compound mepyramine is abolished by adrenalectomy and partially inhibited by β adrenoceptor blockade (Bernauer Hahn and Giertz 1969 Bernauer Gossow and Hahn 1970) The present data emphasize that SRS is a potent bronchoconstrictor substance in the guinea pig Taken together the data suggest that SRS may be of pathophysiological significance in guinea pig anaphylaxis

This study was supported by grants from the Swedish National Association against Heart and Chest Diseases

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Acta physiol scand 1975 94 112–127
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Postnatal Ontogenetic Development of Neurogenic and Myogenic Control in the Rat Portal Vein

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Received 17 December 1974

Abstract

LJUNG B and D STAGE *Postnatal ontogenetic development of neurogenic and myogenic control in the rat portal vein* Acta physiol scand 1975 *94* 112–127

The postnatal ontogenetic development of neuro effector control in vascular smooth muscle of the single unit type has been studied in the rat portal vein Contractile activity was recorded isometrically in isolated preparations from rats 2–38 days of age and in adults rats Spontaneous activity characteristic of the adult portal vein appeared abruptly during the third postnatal week Whereas induced responses to noradrenaline (NA) and acetylcholine (Ach) appeared early during the first week and responses to transmural nerve stimulation occurred at the end of the first week The appearance of spontaneous activity *was accompanied by significant increases in sensitivity to NA (log ED_{50}) and to transmural nerve stimulation* (frequency giving half maximum response) but not to Ach Also maximum respo[illegible] for NA and nerve stimulation relative to Ach responses tended to increase during the first three weeks It is concluded that the development of a mechanism supporting myogenic propagation as revealed by highly synchronized spontaneous contractions is an important factor for promoting the effectiveness of sympathico-adrenergic control in this type of vascular smooth muscle

In recent years the neuro effector function of the isolated portal vein has been investigated in some detail since its properties apparently make it a suitable model for the study of *neurogenic control of propagating vascular smooth muscle (see Ljung 1970 Johansson et al* 1972) The longitudinal smooth muscle in the portal vein of the adult rat exhibits synchronized spontaneous contractions of myogenic origin and propagation (Johansson and Ljung 1967) Such myogenic cell to cell propagation contributes to the effectiveness of the sympathico adrenergic control of this type of smooth muscle by spreading induced activity from muscle cells in the vicinity of the terminal nerve plexus to other non innervated cells located further out in the longitudinal layer This spread of activity is important since noradrenaline (NA) whether released from the terminal nerve plexus or exogenously administered seems to primarily activate the innervated muscle cells in the portal vein (*Johansson and Ljung 1968 Johansson et al* 1970 Ljung 1970 Johansson *et al* 1972 Ljung Bevan and Su 1973)

The media of the portal vein from the adult rat is divided into a thin inner circular layer and a thicker outer longitudinal layer The functionally important terminal vasomotor nerve supply is arranged in a two-dimensional adrenergic plexus between the muscle layers and does not ramify within the muscle tissue (Johansson *et al* 1970 Ljung *et al* 1973) However at birth the double layered smooth muscle configuration of the adult rat portal vein is not found, but develops during the first three postnatal weeks At 10 days of age the p[illegible]ing of the media into two distinct and separate muscle zones the inner circular and the outer longitudinal is clearly distinguishable Unmyelinated nerve fibers are present in the adventitia at birth, but have not been found between the two medial layers of the developing vein when studied in rats up to the 6th week of life (Ts Ao Glagov and Kelsey 1971)

In the light of these histological observations on the developing rat portal vein it appeared to us that the vessel offers the possibility of studying the ontogenesis of neuro-effector function in propagating vascular smooth muscle In the present study the appearance and development of spontaneous activity and induced responses are analyzed and compared in isolated portal vein preparations from young rats The results show dramatic changes in the responses of this vascular tissue during the first few weeks of postnatal life

Methods

Portal vein preparations from 131 young rats (2–38 days of age) and 15 mother rats of the Sprague Dawley strain were studied in these experiments Division of the rats by age weight and length is noted in Table I The animals were killed either by decapitation or by a blow to the neck The portal vein was dissected under a dissection microscope tied at both ends and mounted under longitudinal tension in an organ bath containing 40 ml of Krebs solution (composition see below) Initial resting force exerted on the smooth muscle was adjusted to that length at which optimal active force had been found to be developed in preliminary experiments This preload varied with age between 0 5–4 mN (week 1 and adults, respectively) The average increase in applied passive force was approximately 0 4 mN per week in the young rats The muscle was allowed to stabilize for 60 min and the preload was adjusted during this accommodation period Isometric longitudinal contractile activity was recorded on a Grass polygraph (model 7) by means of a Grass force-displacement transducer (FT 03C) and the force signal was electronically integrated on a separate channel which continuously provided values for mean contractile force Induced responses were calculated as the mean force developed during the period of stimulation minus the mean force during the preceding 3 min control period

The adrenergic nerve plexus of the portal vein preparation was activated by transmural field stimulation (NS) during 30 s periods by use of a Grass Stimulator model S4B which provided square wave impulses of 0 8 ms duration and 15 V in amplitude between platinum electrodes on either side of the preparation In control experiments responses to these stimulation parameters were found to be blocked by tetrodotoxine or adrenergic blocking agents The response of the preparations to different impulse rates ranging from 0 25 to 64 Hz was determined separately for each frequency by starting at the lowest setting and increasing progressively until a maximum response was elicited The interval between each stimulation was at least 10 min to allow time for complete recovery These responses determined for each frequency were then expressed as a percentage of (1) maximum contractile response elicited by noradrenaline (NA) in that particular preparation and ( ) the maximum NS response set at 100 per cent The preparations were divided into groups according to age in weeks and mean values $\pm$S E were calculated for each frequency within the various groups In this way frequency response curves were obtained and plotted at different ages

Dose response curves for exogenously administered NA and acetylcholine (Ach) were determined in the following way The respective drug in appropriate concentration was added directly to the bath for a 3 min exposure period The drug was then washed out by repeated rinses and a 10–15 min interval was

TABLE I Division of rats by age

	Age groups (weeks)						
	1	2	3	4	5	6	A
Age (days)	2–7	8–14	15–21	22–28	29–35	36–38	Adults
Weight (g ±S E)	10±1	24±2	38±2	61±2	96±3	129±4	306±6
Crown-rump length (mm ±S E)	63±2	86±2	106±2	1-8±2	150±2	167±3	234±?
Number	27	30	26	19	18	11	15

allowed for complete return of activity to the baseline level before a 10 times higher drug concentration was given In this way a range of individual responses to drugs administered was determined in concentrations from below threshold up to supramaximum concentrations The experimental values were expressed as a per cent of the maximum response to the respective agent for each preparation Dose response curves for the different age groups were obtained by plotting the response values (mean ±S E) against concentration In addition a calculator program was used to determine the least squares fit of the values from each expt to a true hyperbolic function ED_{50} values were determined from these dose response functions as that concentration at which a 50 per cent response would be obtained and expressed in logarithmic form (log ED_{50})

Maximum peak contractile force for spontaneous activity as measured at the end of the initial accommodation period and for responses to nerve stimulation and exogenous NA and Ach was determined and expressed in absolute values as well as in per cent of that maximum NA response which was elicited in all expts

At the end of each expt the length of the stretched preparation was measured and the wet weight was determined on a Cahn electrobalance after the external fluid had been removed by wiping the tissue against a glass surface until no moist trace was left behind The cross sectional area (mm^2) and maximum tension (mN/mm) were calculated assuming a density of 1 0

Comparison of the mean values between the various age groups was made by use of the Student s t test A statistically significant difference was considered to exist between the compared groups when the p value was less than 0 05 The age of onset (mean ±S E) for a particular response was obtained by sequentially determining the ratio between the number of responding tissues and the total number of tissues each day of life This ratio was considered as a cumulative probability distribution function of time occurring during the transition period from complete unresponsiveness to the day that all tissues responded

The Krebs solution had the following composition in mM NaCl 12? KCl 4 73 $NaHCO_3$ 15 5 KH PO_4 1 19 MgCl 1 19 $CaCl_2$ 2 49 Glucose 11 5 and $CaNa_2$-versenate 0 026 It was continuously bubbled with 4 per cent CO_2 in O_2 to maintain a pH of 7 35 at the thermostatically set temperature of 38 C

The drugs used were l noradrenaline bitartrate (nor Exadrin conc Astra AB Södertälje Sweden) acetylcholine chloride (Merck West Germany) and tetrodotoxin (Sigma Chemical Co St Louis Mo) Freshly prepared saline diluted drugs in a volume of 0 4 ml were injected directly into the 40 ml bath to give the desired concentration

Results

Onset of portal vein responses in neonatal rats The histograms in Fig 1 illustrate the age of onset for spontaneous activity and responsiveness to exogenously administered noradrenaline (NA) acetylcholine (Ach) and to transmural field stimulation of the nerve supply (NS) in the isolated portal vein of the neonatal rat No spontaneous activity was found during the first 2 weeks of postnatal life In a transition period between the 15th and 18th days of age spontaneous activity appeared in an increasing number of vessels so that after the 19th day it was evident in all preparations The average age of onset was 16 5 ± 1 6 (days ±S E) Portal vein responses induced by exogenous NA as well as by Ach occurred much earlier at

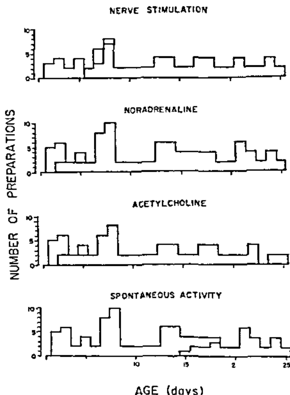


Fig. 1 Age of rats at onset of portal vein responses Number of preparations exhibiting spontaneous activity and responding to exogenous acetylcholine exogenous noradrenaline and to transmural nerve stimulation (filled in squares) and number of those not responding (open squares) plotted against age (days) Note early appearance of induced portal vein responses in comparison with later onset of spontaneous activity

an average age of 3 7 ± 1 3 (days ± S E) Transmural field stimulation induced neurogenic responses of the portal vein from the 6th day on with an average age of onset of 6 1 ± 1 3 (days ± S E) Thus portal vein responsiveness to NA Ach and nerve stimulation was not a consistent finding during the first week of postnatal life but after the 8th day of age all portal vein preparations responded to all three stimuli

The development of spontaneous activity and induced responses to NA Ach and NS in the portal vein of maturing rats is described in the following sections with regard to maximum force and sensitivity to the stimulating influences

Development of spontaneous activity As illustrated in Fig 1 no spontaneous activity was recorded in the isolated portal vein of rats during the first 2 weeks of postnatal life Between 15 and 18 days of age spontaneous phasic activity first appeared and was characterized by erratic contractions of variable amplitude occurring at irregular intervals (Fig. 2 left diagram) During this transition period 8 out of the 16 preparations exhibited this type of spontaneous myogenic activity as recorded at the end of the initial 1 h accommodation period In an additional 4 out of these 16 preparations phasic activity appeared at the end of the NA and/or Ach experiments even though the drug was apparently removed by repeated rinses Phasic contractile activity of low frequency occurred in a few preparations from younger rats (13 to 15 days) that were allowed to stand overnight in a bath of normal Krebs solution After 19 days of age all portal vein preparations exhibited spontaneous

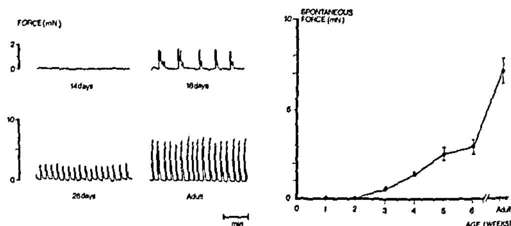


Fig 2 Development of spontaneous activity in rat portal vein Recordings illustrating pattern of spontaneous activity at different stages of development (left diagram) Spontaneous force (mean ± S E) plotted against age in weeks (right diagram) For number of observations see Table 1 Note gradual increase in mean force with advancing age

myogenic activity Gradually a more consistent pattern of regular phasic synchronized activity was established so that by the third to fourth week regular phasic contractions of comparable magnitude and at a rate of approximately 2-4 per min occurred throughout the entire experiment (Fig 2 left diagram)

The mean force exerted by spontaneous contractile activity as measured at the end of the initial accommodation period progressively increased with age from zero during the first two weeks up to nearly 3 mN by the end of the sixth week This latter value was about half of the spontaneous force exerted by the adult portal vein (Fig 2 right diagram) The peak force of spontaneous activity and of induced responses was expressed as a percentage of the maximum force developed in response to NA in each experiment so that comparison could be made between the magnitudes of maximum responses to different exogenous stimuli at different ages This percentage of spontaneous activity increased from zero during the first two weeks up to 1/3 of maximum NA response during the 4th to 6th weeks and was half of the maximum NA response in adult rats (see Fig 8) Thus the basal level of spontaneous activity as expressed in terms of maximum contractile force increased with age in the developing rat portal vein

The synchronized spontaneous contractions were not affected by complete blockade of the α adrenergic receptors or by neuronal blockade with tetrodotoxin In Fig 3 at the left spontaneous activity and a response to transmural nerve stimulation (4 Hz) are illustrated before tetrodotoxin block After a 30 min exposure to tetrodotoxin (10 ⁻⁶ M) the frequency and amplitude of the spontaneous contractions were unchanged whereas the nerve response was completely blocked Sixty min after the drug had been washed out the spontaneous activity remained the same and the nerve response returned to its previous level

In the following sections it will be seen that the onset of spontaneous activity after 2 weeks of age coincided with a marked increase in sensitivity to exogenous NA and to vaso-



Fig 3 Recordings of spontaneous activity and responses to transmural stimulation (4 Hz 0 8 ms and 15 V) in a 2 day old rat portal vein preparation before (left panel) 30 min after (middle panel) the administration of tetrodotoxin (10^{-6} M) and 1 h after wash out of the drug (right panel) Note that the response to transmural stimulation is blocked by tetrodotoxin while spontaneous activity is unaffected

motor nerve stimulation This age apparently represented a milestone in the maturation of the rat portal vein

Portal vein response to noradrenaline Actual tracings from experiments on the rat portal vein before and after the appearance of spontaneous activity (8 and 21 days of age respec tively) and on a preparation from a mother rat illustrate the contractile responses to exogenous NA (Fig 4) The response pattern in the young portal vein without spontaneous activity was characterized by a smooth increase in force from baseline up to a plateau level which persisted throughout the exposure period The first response above threshold was often comparatively great in magnitude Subsequent 10-fold increases in NA concentration often gave only small additional increases in amplitude After the appearance of spontaneous activity (middle and lower recordings) the pattern of the contractile responses was altered First at low NA concentrations the frequency of the phasic contractions increased Then the

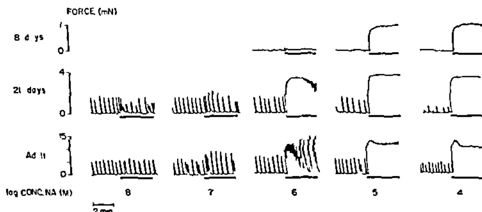


Fig 4 Recordings of myogenic acti ity and e c tatory responses to noradrenal ne (NA) in portal vein preparations from rats 8 and 21 days of age respectively (upper and m ddle pan ls) and an adult rat (lower panel) Agonist adm nistered in progressively increas ng concentrations (10^{-8} to 10^{-4} M) d ring 3 m n periods as ind cated by bar length Note change in NA sens tiv ty and in response pa tern after appearance of spontaneous activity

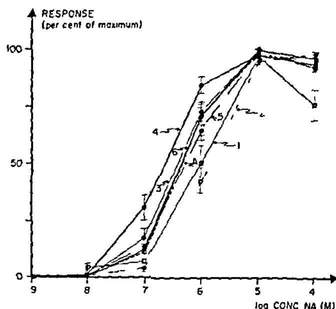


Fig 5 Relationships between noradrenaline (NA) concentration and integrated force response expressed as per cent of maximum response (mean ± S E) in portal vein preparations from young rats grouped according to age in weeks (1–6) and from adult rats (A) Number of observations in each group 1 – 14 2 – 18 3 – 16 4 – 15 5 – 12 6 – 7 A – 9

amplitude of these contractions also increased above the basal level (NA conc 10 ⁷ M) The next stage was one of incomplete tetanus characterized by the rapid development of a smooth increase in force which gave way to phasic contractions of augmented amplitude The final stage resembled a complete tetanus marked by the rapid development of force which plateaued at a stable level throughout the exposure period This last tetanic stage rred at a NA concentration of 10^{-5} M Thus the progression of development of responses a concentration dependent manner appeared more gradual in those preparations with spontaneous activity

All responses to NA were quantitated as the increase in mean active force (see Methods) and expressed as a percentage of the maximum NA response Dose response relationships for NA (10 to 10 ⁴ M) were determined in portal vein preparations from 89 young rats (2–38 days of age) and in 9 adult rats These 98 tissues were then grouped according to age in weeks (Table I) and mean integrated response values ± S E were determined for each NA concentration (Fig 5) Except for week 4 which was furthest to the left hardly any variation was found between the dose response curves after 3 weeks of age Those of the first two weeks were displaced to the right Thus in order to elicite a given response a higher NA concentration was required in these youngest rats Furthermore it was seen from Fig 5 that maximum responses were obtained at a NA concentration of 10 ⁵ M

In an additional 48 expts where other means of stimulation were primarily employed NA in the concentration of 10 ⁵ M was also administered so that maximum active force in response to NA was measured in a total number of 146 preparations This maximum force increased progressively with age from a mean value of 0 3 up to 9 mN (weeks 1 and 6 respectively) reaching a peak value of 13.5 mN in adult rats (Fig 6) The differences between these maximum force values were statistically significant for all age groups The cross sectional area of each preparation was calculated from its measured length and weight

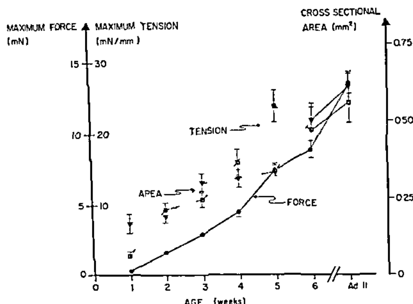


Fig 6 Maximum force response to exogenous noradrenaline (●—●) cross sectional area (▼- -▼) and calculated maximum tension (□---□) in portal vein preparations from rats at different ages Mean ±S E indicated for each age group For number of observations see Table I

assuming a density of 1 0 (see Methods) Probably due to the technical difficulties involved in obtaining these measurements in small tissues statistically significant differences for the mean values of cross sectional area were not detected between all age groups However the trend was that area increased with age from a mean value of 0 17 mm² (week 1) up to 0 62 mm² (adults) (Fig 6) The values for maximum active force and cross sectional area, albeit crudely determined allowed for the calculation of maximum tension Again although a definite trend of increase with age was seen from a mean value of 3 mN/mm² (week 1) up to 24 mN/mm² (adults) statistical differences between all age groups were not obtained

The tissue sensitivity to NA was determined as the ED value in each dose response experiment (see Methods) Means of the log ED_{50} values were calculated for each week (Fig 7) A distinct increase in NA sensitivity occurred after the 2nd week *i e* that concentration of NA required to produce a 50 per cent response decreased All mean log ED_{50} values from the 3rd week onto adults were significantly lower than those of the 1st and 2nd weeks The mean log ED_{50} value for the 4th week was lowest when compared with all other age groups As mentioned above the increase in ED_{50} for NA after the 2nd week occurred simultaneously with the appearance of spontaneous activity (Fig 7)

Portal vein response to acetylcholine Exogenous Ach caused excitatory responses of the portal vein from the 3rd day on (Fig 1) The different patterns of contractile activity induced by graded concentrations of Ach at the different stages of portal vein maturity resembled the NA responses illustrated in Fig 4 The progression of Ach induced responses with increasing Ach concentration was more gradual than for NA

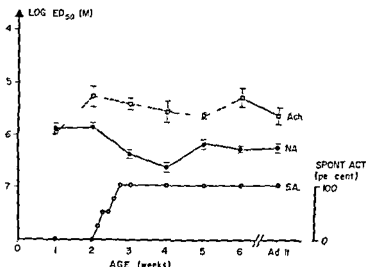


Fig. 7 Log ED_{50} values (mean ± S E.) obtained from a least squares fit of integrated response values for noradrenaline (NA) and acetylcholine (Ach) to true hyperbolic function in developing rat portal vein (upper curves) Lower curves represent percentage of all preparations displaying spontaneous activity (S A) at different ages Number of observations for NA see Fig. 5 Ach week 1 = 4 2 = 6 3 = 10 4 = 4 5 = 4 6 = 4 adult = 4 and S A. see Table 1 Note the significant decrease in log ED_{50} for NA but not for Ach, for all age groups after week 2 when spontaneous activity appeared

Dose response relationships for exogenously administered Ach in concentrations ranging from 10^{-8} to 10^{-3} M were determined in 38 young rats (2–37 days of age) and in 4 adult rats Tissue sensitivity to Ach was determined as the ED_{50} value in each dose response experiment (see Methods) Mean log ED_{50} values were calculated and grouped according to age in weeks (Fig. 7) In comparing Ach with NA the portal vein was less sensitive to Ach in that significantly higher Ach concentrations were required to produce a 50 per cent response in all age groups except week 1 From the second week on, the sensitivity of the young portal vein to Ach did not change with advancing age

The mean values for maximum Ach response increased progressively from 0 3 up to 9 7 mN (weeks 1 and 6 respectively) reaching the value of 15 9 mN in adult rats The differences between these increases were statistically significant for all age groups Mean values of maximum force for Ach expressed as a percentage of the maximum response to NA 10^{-5} M in each experiment are illustrated in Fig 8 The maximum Ach response was significantly greater than that for NA during the first two weeks but very close to the NA value from the third week on

Portal vein response to nerve stimulation Actual recordings from transmural stimulation experiments on isolated portal veins from rats of different ages are illustrated in Fig. 9 In the youngest rats responses were obtained only at high impulse rates and after a considerable delay sometimes lasting longer than the 30 s stimulation period The pattern of contractile response in these preparations without spontaneous activity resembled the NA response in that there was a smooth increase in force from baseline up to a peak level Following stimulation at high frequencies there was often a biphasic decrease in force (Fig. 9 top recordings) When the spontaneous activity appeared after the second week

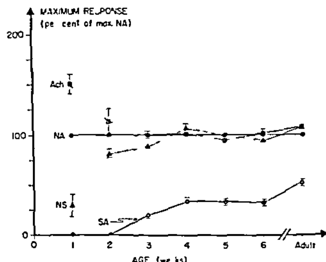


Fig 8 Maximum force (mean ±S E) responses for spontaneous activity (SA) exogenous noradrenaline (NA) acetylcholine (Ach) and transmural nerve stimulation (NS) expressed as a percentage of max NA in portal veins from rats at different ages Number of preparations for SA see Table I Ach see Fig 7 NS see Fig. 10

neurogenic responses were obtained at much lower stimulation frequencies and occurred nearly instantaneously with the stimulation At low and moderate nerve impulse frequencies the phasic contractions increased in rate and amplitude At high frequencies they tended to fuse giving a tetanus-like response (Fig 9 middle and lower recordings) Maximum force occurred at an impulse frequency of 32 Hz in both young and adult rats

Curves relating neurogenic response expressed as a percentage of the maximum NA response to stimulation frequency were determined in 101 young rats (2–38 days of age) and in 11 adult rats The mean response values ±S E were plotted according to age in weeks (Fig 10 left diagram) During the first two weeks the responses as related to maximum NA response were very small and elicited only after exceedingly high non physiologic impulse rates Thereafter the threshold frequency was markedly lowered and from the third week on the relative amplitude of neural responses was higher without obvious variation between the different age groups Maximum responses were elicited by a frequency of 32 Hz in all age groups

As for NA and Ach the amplitude of the maximum NS response increased progressively with age from a mean value of 0 1 up to 8 mN (weeks 1 and 6 respectively) reaching a peak value of 14 mN in adult rats The differences between these mean values were statistically significant for all age groups except between weeks 5 and 6 Mean values of the maximum neurogenic force expressed as a percentage of the maximum NA response in each experiment are shown in Fig 8 The mean maximum NS response was significantly less than that for NA during the first 3 weeks but not different from NA and Ach values from the 4th week on

The frequency response curves of Fig 10 left diagram indicate that not only the maximum amplitude but also the sensitivity to change in nerve impulse frequency varied with age In Fig 10 right diagram this is more clearly illustrated when the neurogenic responses are expressed as a percentage of maximum NS responses and related to stimulation frequency on a linear scale The curves for the 1st and 2nd weeks were clearly displaced to the r

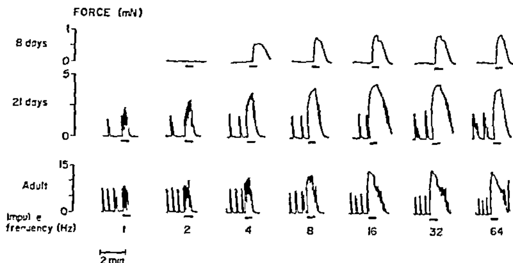


Fig. 9 Tracings of responses to transmural nerve stimulation at graded frequencies (values at bottom) (0 8 ms, 15 V) in portal vein preparations from rats 8 and 21 days of age respectively (upper and middle panels) and from an adult rat (lower panel) Stimulation applied for 30 s intervals indicated by bar length Note changes in low frequency sensitivity in response pattern and in delay time for onset of response after appearance of spontaneous activity

of the curves for older age groups which in turn showed little variation Thus a higher frequency was needed to elicite a given relative response in the youngest rats The frequency required to give a 50 per cent response can be estimated by inspection of the curves in Fig. 10 right diagram Thus value was 13 and 7 Hz for weeks 1 and 2 respectively and

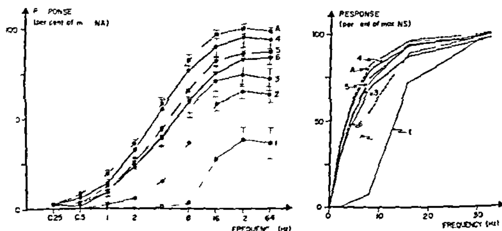


Fig. 10 Relationship between nerve stimulation frequency and integrated contractile response expressed as a percentage (mean ± S E.) of maximum response to exogenous noradrenaline (max NA) in left diagram and expressed as a percentage of maximum neurogenic response (max NS) at right. Portal vein preparations from young rats grouped according to age in weeks (1–6) and from adult rats (A). Number of observations in each group 1 = 5 2 = 23 3 = 20 4 = 15 5 = 14 6 = 7 A = 11 Note that sensitivity to nerve stimulation increases during the first 3 weeks.

ranged between 3 2 to 4 6 Hz for the older age groups This indicates a definite increase in sensitivity to NS after the 2nd week which appeared coincidently with the onset of spontaneous activity and the increase in NA sensitivity (see Fig 7)

Discussion

The longitudinal muscle layer of the portal vein from various adult laboratory animals exhibits spontaneous phasic activity when studied *in vitro* The synchronization of the elect rical activity within the tissue which is required for effective coordinated contractions has been demonstrated in the adult rat portal vein to be due to myogenic cell to-cell propagation (Johansson and Ljung 1967 Ljung och Stage 1970)

In the isolated postnatal rat portal vein passively stretched according to age to give optimal active force phasic activity first appeared during the third week of life These spontaneous contractions were not affected by exposure to tetrodotoxin in concentrations which eliminated nerve responses (Fig. 3) and thus they are in all probability dependent on myogenic mechanisms The apparent lack of single unit behavior during the first two postnatal weeks probably reflects the absence of effective myogenic spread of excitation in the very young portal vein Developing smooth muscle cells grown in tissue culture have been found to form aggregates which begin to contract in synchrony as the cells become electrotonically interconnected (Purves Mark and Burnstock 1973) Likewise it seems reasonable to surmise that the appearance of synchronized activity in the developing smooth muscle of the rat portal vein at around the 15th day might reflect the establishment of functioning bundles of coupled muscle cells

However in addition to myogenic spread of excitation coordinated phasic contractions require activity that is rhythmically induced from groups of muscle cells with pacemaker function Such activity can be evoked and/or accelerated in the adult portal vein by depolariz ing influences such as exogenous NA (Ljung and Stage 1970) or stretch (Johansson and Mellander 1975) In the present experiments coordinated phasic contractile activity was sometimes induced at an age of 15–18 days but never during the first two weeks of life regardless of the degree of stretch

It is of great interest that in the third week of life coincidental to the appearance of spontaneous activity the sensitivities to exogenous NA and to transmural nerve stimulation were found to increase significantly In a series of previous studies it has been shown that the ability of the smooth muscle of the adult rat portal vein to support propagation contrib- utes in an important manner to the effectiveness of the sympathico-adrenergic control of the vessel During nerve activity the released transmitter substance NA apparently reaches only smooth muscle cells near the terminal adrenergic nerve plexus in the high peak con centrations which determine the neurogenic responses in the adult rat portal vein (Ljung 1969 1970 Johansson *et al* 1972) In addition the functionally important α receptors seem to be mainly restricted to these comparatively few innervated cells (Johansson *et al* 1970 Ljung *et al* 1973 Bevan and Ljung 1973) Therefore myogenic propagation of locally induced excitation is needed for the recruitment of the succeeding non-innervated

smooth muscle cells to respond effectively not only to nerve stimulation but also to exogen ous NA As a consequence the increased sensitivity to adrenergic stimulation found after the second week can probably be attributed to the development of functionally important myogenic propagation mechanisms in the maturing vascular muscle

It appears that such an increase in functionally relevant spread of activity within vascular smooth muscle does not necessarily parallel the relative abundance of intercellular connec tions or the passive electrical properties of the muscle tissue In a series of studies (Mekata 1971 1974 and Mekata and Niu 1972) it has been shown that the smooth muscle of elastic arteries has cable properties and length constants of considerable magnitude However action potentials cannot be triggered (see Mekata 1974) and it seems that the lack of such an efficient regenerating mechanism may account for the apparent absence of longitudinal spread of induced activity in large arteries This is in contrast to the situation in the adult portal vein and peripheral arteries where functional spread of induced activity has been demonstrated (Bevan and Ljung 1974) Thus it seems possible that the occurrence of spontaneous activity and the concomitant increase in sensitivity to α receptor stimulation might reflect a change in membrane excitability and/or the establishment of muscle cell interconnections in the developing portal vein

In contrast to adrenergic stimulation the sensitivity to exogenous Ach was not found to increase during development The results of a recent study indicated that as opposed to the sensitivity to α adrenergic stimulation the sensitivity to non adrenergic influences such as Ach and K_+ is evenly distributed within the smooth muscle (Bevan and Ljung 1973) Thus it may be concluded that myogenic propagation in this type of vascular smooth muscle is less important in promoting the effectiveness of cholinergic than of adrenergic responses

The greater sensitivity of the maturing smooth muscle to α adrenergic stimulation might course be due to alterations in the properties of α receptors or increased reactivity of the ar processes linking the agonist receptor interaction to the contractile response How ever previous studies of the ontogenetic development of both α and β adrenergic and cholin ergic receptor function in isolated tissues without spontaneous activity have shown that the tissue sensitivity to the agonists NA and Ach did not change with maturation (Mc Murphy and Boréus 1968 Boreus and McMurphy 1971 Boreus Malmfors McMurphy and Olson unpubl results) This further supports our thesis that it is the appearance of efficient myogenic propagation in the developing portal vein which is responsible for the increased sensitivity to NA

The maturation of the smooth muscle of the portal vein was not only reflected by the development of spontaneous activity and by the alteration in sensitivity to exogenous NA but also by a progressive increase of maximum force per unit cross sectional area exerted spontaneously and in response to exogenous NA and Ach with advancing age (*cf* Fig 2, 6 and 8) The latter was not dependent upon myogenic propagation but seemed to reveal a structural or functional alteration in the smooth muscle itself It may be that the ratio be tween smooth muscle content and the total cross sectional area of the vessel increases during the entire period studied or that each unit of smooth muscle becomes more efficient in generating force At present sufficient morphological information is not available to settle this question However the neonatal rat portal vein has been studied with electron

microscopy (Ts Ao *et al* 1971) It was found that the adult pattern of a double layered muscular structure with division into inner circular and outer longitudinal smooth muscle layers was not present at birth but developed during the first 3 postnatal weeks Partitioning of the media was found to start at 3 days of age A clear orientation of the two muscle zones separated by an intervening fibrous layer was established by 7–10 days and was further differentiated in the ensuing two weeks

A remarkably similar time course for the development of skeletal muscle of the neonatal rat hindlimb into two functional types fast and slow muscle has been observed to occur during the first 3 weeks of postnatal life An increase in sensitivity of vascular smooth muscle to NA occurred simultaneously so that by the time of weaning at about 21 days the reactivity to exogenously administered NA was almost the same as in the adult rat (Gray 1972)

Responses to nerve stimulation at high frequencies first appeared towards the end of the first week of life These responses which could be blocked pharmacologically (see Fig 3) demonstrated the presence of functioning adrenergic nerves at this age The sensitivity to transmural nerve stimulation increased considerably during the first two weeks as apparent from the shift of the frequency response curves to the left (Fig 10 right diagram) and by the increased response amplitude relative to the maximum NA response (Fig 10 left diagram and Fig 8) In addition the considerable delay existing between onset of nerve stimula tion and response was greatly shortened after the second week (see Fig 9) The outgrowth of nerves appears to be particularly important for the observed increases in neural responses between the first and the second week In this period of time the maximum response to nerve stimulation expressed as a percentage of max NA was almost doubled and the fre quency giving 50 per cent of the maximum NS response was approximately halved After the second week the development of myogenic propagation must be an important factor for the enhancement of the neurogenic responses In fact the importance of this mechanism in the case of nerve induced activity must be at least as great as it is for exogenous NA During physiological nerve impulse rates the released transmitter will apparently reach only the nearest muscle cells in effective concentrations (Johansson *et al* 1972) Therefore myogenic propagation is required for normal activation of the non innervated muscle cells

In the thin wall of the young portal vein without spontaneous activity the released trans mitter may accumulate during prolonged stimulation at high frequencies so that weak responses were elicited after long delays After the appearance of spontaneous activity there was hardly any delay and the sensitivity to NA in the physiological frequency range (less than 8 Hz) was greatly increased

Another functional aspect of the developing nerve supply is the appearance of an efficient neuronal uptake of NA (*cf* Sachs *et al* 1970) In the adult portal vein this uptake mechanism greatly influences the responses to exogenous NA so that a considerable prejunctional supersensitivity is obtained after denervation (Johansson *et al* 1970) In the present experi ments the importance of this mechanism has not been studied in detail but it seems to be a possible explanation for the observed decrease in NA sensitivity after the 4th week (see Fig 7)

The morphological development of the adrenergic innervation has been studied in different

organs of the rat (de Champlain *et al* 1970 Owman Sjoberg and Swedin 1971) However the ontogenesis of the neural supply of the portal vein is not known in detail as yet In the adult rat the functionally important terminal nerve plexus is located between the longitudinal and circular muscle layers (Johansson *et al* 1970 Ljung *et al* 1973) Ts Ao *et al* (1971) found unmyelinated nerve fibers in the adventitia at birth but they did not detect any nerve structures between the two muscle layers in the rat portal vein up to 6 weeks of age The time course for the development of this adult pattern of innervation awaits further study

The hemodynamic consequences resulting from pronounced activity of this single unit type of longitudinally arranged spontaneously active smooth muscle of the portal vein are not known In the previous studies from this laboratory the adult portal vein has been used as an *in vitro* model for vascular smooth muscle of the single unit type *i e* the kind present in the hemodynamically all important precapillary resistance and sphincter vessels Study of the developmental aspects of the young rat portal vein may be important for an understanding of the ontogenesis of single unit vascular smooth muscle in general However it might rather reflect a structural and functional postnatal adaptation to the altered cardiovascular situation occurring in the portal circulatory system after birth or secondary to maternal endocrine influences

In the human fetus the portal vein receives only little blood flow from the inactive gastrointestinal tract There is a direct communication between the umbilical vein and portal sinus which drains into the caval vein *via* sinus venosus Immediately after birth the umbilical circulation ceases and the umbilical and portal vein blood pressures fall rapidly Several hours later when the gastrointestinal tract begins to function portal vein blood flow increases considerably to cause a marked widening in portal vein diameter during the first 3 weeks of life This increased diameter is associated with an unfolding of longitudinal muscle (Meyer and Lind 1966) In the rat use of the whole body freezing technique has likewise demonstrated that significant increases in the inner diameter of the postnatal portal vein occur at birth and during the first week of life (Marsk 1972)

In experiments on the isolated portal vein from animal species which are more mature than the rat at birth it has been found that spontaneous activity and neurogenic responses appear in the newborn (Stage unpublished) Thus it appears that the postnatal development of the rat portal vein during the first three weeks of life occurs prenatally in other more mature animals Therefore it is likely that the observed vascular maturation is independent of the hemodynamic or endocrine changes at birth

In conclusion a marked structural and functional differentiation occurs in the developing portal vein during the neonatal period of life When this differentiation is complete the portal vein has fully developed a single unit type of longitudinal smooth muscle capable of coordinated phasic contractile activity Once myogenic propagation is established the effectiveness of sympathetic nervous control is greatly enhanced by coordinating the contraction and by recruiting non innervated smooth muscle cells

Thanks are due to civil engineer Lars Stage for designing the electronic equipment and computer programs to Mrs Ulla Axelsson and Miss Ann Kjellstedt for able technical assistance and to Miss Lena Nimmerfors for secretarial aid

Supported by grants from the Swedish Medical Research Council (3884) from Magn. Bergvalls Stiftelse and from the Medical Faculty University of Goteborg.

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Influence of Amphetamine Sulphate on Cerebral Blood Flow and Metabolism

By

CHRISTER CARLSSON MAGNUS HAGERDAL AND BO K SIESJÖ

In the course of a study of the effect of hyperthermia upon cerebral metabolic rate for oxygen (CMR_O) and cerebral blood flow (CBF) we have investigated separately other procedures that may increase CMR_O including administration of amphetamine sulphate Since the results have disclosed a hitherto unknown effect of amphetamine on CBF and CMR_O a preliminary account of the results is given

Methods

Unstarved male Wistar rats were anesthetized with 2–3 % halothane immobilized with tubocurarine chloride (0 5 mg kg^{-1}) i v tracheotomized and ventilated on 70 % N_2O and 30 % O_2 Halothane was withdrawn at the time of tracheotomy A femoral artery was cannulated for blood pressure recording and for sampling of arterial blood The superior sagittal sinus was exposed by a small burr hole for sampling of cerebral venous blood CBF was measured according to the Kety and Schmidt principle using the ^{133}Xenon method described in a previous communication (Norberg and Siesjo 1974) CMR_O was calculated as the product of CBF and the arteriovenous difference in oxygen content (AVD_O) In a few experiments repeated measurements were performed of AVD_O prior to and following administration of amphetamine in others cortical blood flow was measured 2 and 5 min after the drug was given using the ^{14}C-ethanol method described by Eklöf *et al* (1974)

Results

In all experiments to be reported the body temperature was close to 37 C the Pa_{CO_2} was close to 40 mm Hg and the Pa_{O_2} exceeded 100 mm Hg In 8 expts CBF and CMR_O were measured 30 min after the *i p* administration of amphetamine sulphate in a dose of 5 mg kg^{-1} The results were compared to those obtained in 12 uninjected animals Table 1 shows that there was a highly significant increase in CMR_O by about 40 per cent Somewhat unexpectedly CBF increased to an average value of 400 per cent of normal an increase which cannot be explained by the small increase in blood pressure In other experiments the AVD_O was found to decrease to less than 50 per cent of normal already at 1 and 2 min following the i v administration of amphetamine in a dose of 2 5 mg kg^{-1} Measurements of regional CBF using the ^{14}C-ethanol method confirmed that CBF increased about threefold after 2 and 5 min

TABLE I Cerebral blood flow (CBF) and cerebral metabolic rate for oxygen (CMR$_O$) in rats 30 min after administration of amphetamine sulphate in a dose of 5 mg kg^{-1} i p Also given are mean arterial blood pressure (MABP) and arterial P_{CO} The values are means ± S E

Exp group	MABP	P_{aCO}	CBF	AVD$_O$	CMR$_O$
Control	139	37 9	110	9 4	10 2
(n = 1[illegible])	±4	±0 7	±4	±0 4	±0 2
Amphetamine sulphate	163	40 1	4 8	3 6	14 3
(n = 8)	±3	±1 0	±45	±0 3	±0 6
$p < 0.001$					

The increase in CBF after amphetamine was unrelated to changes in CO_2 tension Furthermore studies of tissue metabolites revealed that the tissue contents of lactate and pyruvate remained unchanged Thus tissue acidosis could not have been responsible for the hyperemia

Discussion

Previous observations in man (Abreu et al 1949 Shenkin 1951) have given no indication that amphetamine significantly affects CBF or CMR$_O$ Recently Nahorski and Rogers (1973) reported that amphetamine sulphate administered to rats in a dose of 5 mg kg^{-1} i p caused a decrease in cerebral metabolic flux during the first 30 min period and a subsequent increase thereafter The method used by the authors was an indirect one and it is not known if it gives a valid measure of metabolic rate under the conditions of the experiments

The present results demonstrate that CMR$_O$ increases by about 40 per cent 30 min after i p administration of amphetamine sulphate and that there is a severalfold increase in CBF When CMR$_O$ is decreased by barbiturate anesthesia of hypothermia or increased by hyperthermia the relative changes in CBF and CMR$_O$ are similar Thus the increase in CBF after amphetamine administration is far in excess of the elevation in metabolic rate This indicates that amphetamine has a more direct effect on the unknown mechanisms that normally adjust the rate of blood flow to the functional activity

The authors are grateful to Miss Gunilla Gido for her skilled technical assistance This study was supported by grants from the Swedish Medical Research Council (projects No 14X 263 and 14X 2179) from the Swedish Bank Tercentenary Fund and from U S PHS Grant No 5 R01 NS07838-06 from NIH

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α-γ-Linkage in the Spinal Generator for Locomotion in the Cat

By

A SJOSTRÖM and P ZANGGER

Grillner and Zangger (1974) reported that after a complete transection of all dorsal roots preparations spinalized at lower thoracic or upper lumbar levels can still produce rhythmic locomotor like activity with an alternate activation of flexors and extensors provided that they have been injected with DOPA DOPA and Nialamide Clonidine i v or that the transection of the spinal cord is chronic The present study was undertaken to see whether or not γ motoneurones to the appropriate muscles are coactivated with the α motoneurones during this rhythmic activity

The γ motoneurone activity has been recorded in the form of single γ efferents isolated in peripheral nerve filaments and identified by their conduction velocity from the ventral roots (see Bergmans and Grillner 1969) to either a flexor (tenuissimus) or an extensor (lateral gastrocnemius) Also muscle spindle afferents (primary endings) identified conventionally (cf Matthews 1972) were recorded from a flexor (anterior tibial) and an extensor (soleus) The preparations were decerebrated and spinal (Th 12 to 13) without anesthesia from 2 h prior to the beginning of the recording period All dorsal roots were transected and 50 mg/kg Nialamide was injected i v followed by up to 50 mg/kg DOPA After this injection spontaneous rhythmic activity (0 5–1 2 Hz) could occur but during uni or bilateral continuous stimulation (10–200 Hz) of the cut dorsal roots (L 5 or L 6) the rhythmic efferent activity increased in frequency and became more regular When recording γ-efferents the preparations were curarized and α and motoneurones were recorded in filaments when recording primary endings a strain gauge was connected to the muscle under investigation The length *of the muscle was measured in relation to the maximal length and the corresponding joint* angle (Grillner and Udo 1971)

Fig 1 A shows the discharge in a primary ending from a soleus spindle and the marked acceleration during each period of extrafusal contraction For comparison (B) is shown the typical unloading of the ending occuring when the nerve is stimulated with a single shock The graph in D shows the rhythmic increase in discharge frequency in a single extensor γ motoneuron The bars below show that the periods during which the α motoneurones are accelerated coincide with the increased γ activity Therefore it is no doubt that the results shown in A is due to α -coactivation In C is shown that α and motoneurones to a flexor muscle are also coactivated

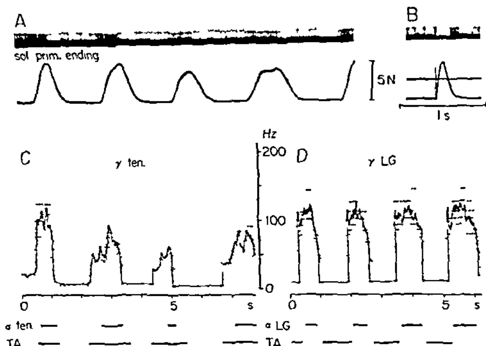


Fig. 1 Rhythmic α γ-co ct vation to flexors and extensors *A* shows the acti ity of a single *primary (con* duction velocity 85 m s) ending from an ankle extensor (m soleus) recorded in a dorsal root filament L 7 (upper trace) during rhythmic isometric contraction of the muscle (tension lower trace) The *muscle* was held at a length corresponding to 60 ankle flexion resulting in a resting discharge of 36 Hz. *Continuous* bilateral stimulation of L 6 dorsal roots (100 Hz 60 μA squarewave pulses of 0 2 ms durat on) In *B* is shown a twitch contract on after stimulation of the muscle nerve obtained just before the recordings *in A* *C* shows the frequency of discharge (ordinate) in a single flexor γ-efferent (conduction velocity 35 m s) during spinal stepping recorded from a tenuissimus nerve f lament The recorded *sequence* was *taken just* after termination of a period of ipsilateral continuous stimulation of the dorsal root L 6 Dots mark the instantaneous frequency (1/interval length) the curve is plotted with a floating average *over the 8 nearest* intervals The horizontal bars below the graph indicate the duration of the acti ity of a single α-e ferent recorded in the same filament (α ten) and the acti ity in a nerve to an ankle flexor (TA tibialis *an erior)* The resolution for the frequency plot was l mited by the input device to the computer (digitizer table) D shows rhythmic discharges of a single extensor γ efferent (conduction elocity 29 m s) recorded in a lateral gastrocnemius (LG) nerve filament during ipsilateral stimulation of L 5 dorsal root (100 Hz, 30 μA) Presentation as in C The bars indicate the period of acti ity in a single α-efferent recorded in the same nerve filament (α LG) Note t me and tension (100 g wt. equals 0 981 N) cal bration for A and B

Hence it can be concluded that the central spinal mechanism that g nerates this rhythmic locomotor activity in fact is coactivating α and motoneurones i e there is a central spinal α linkage This is in agreement with the predictions made from the studies of α linkage in the late discharges occuring after DOPA (Grillner 1969 see also Perret 1973) The entire pattern of muscle activity in locomotion can be generated in the low spinal animal (Grillner 1973) and it thus appears that these movements are α γ linked and it follows that the findings of α-γ-coactivation in high decerebrate walking cats should also be due to a spinal mech anism (Severin *et al* 1967)

The helpful d scuss ons with and the valuable crit ci m f om Dr Sten Gr lln s greatfully cknow ledged.

This work was supported by a grant from the Swedish Medical Research Council (14X 30 6) and a grant from the Medical Faculty in Goteborg P Zangger was supported by the Swiss National Foundation. The skilful assistance of Mrs M Svanberg is gratefully acknowledged.

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Brown Adipose Tissue and the Significance of the Venous Plexuses in Pinnipeds

By

ARNOLDUS SCHYTTE BLIX HANS J GRAV and KEITH RONALD

Anatomically the cardiovascular system of Pinnipeds differs in important respects from that of most other mammals in the occurrence of spectacular venous plexuses (Burow 1838) These plexuses (Fig 1 a) which are localized in the neck region on the pericardium on the abdominal wall adjacent to the kidneys and as a stellate renal plexus have been described in detail by Harrison and Tomlinson (1956) and by McCarter (1974) In contrast to this comprehensive anatomical documentation the physiological significance of the plexuses have remained obscure During recent field studies (in Eastern Canada and off East Greenland) however we have discovered that the venous plexuses of both harp seals and hooded seals appear to be embedded in brown adipose tissue

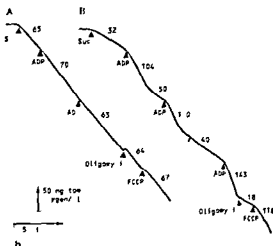
It is generally agreed that brown adipose tissue possesses a decisive thermogenic potential (Smith and Horwitz 1969) When activated the brown adipose tissue produces heat by oxidation of its lipid stores This oxidation proceeds at a high rate by virtue of mitochondria in which oxygen uptake and energy conservation are loosely coupled processes (Christiansen *et al* 1969) With mitochondrial respiratory control thus suspended this principle allows the tissue to sustain a high rate of respiration and hence of heat production irrespective of whether phosphate acceptor (ADP) is available to the mitochondria

In separate series of experiments we have confirmed that mitochondria isolated from the brown adipose tissue of harp seals behaved as would be expected of loosely coupled mitochondria (Fig 1 b) The light and electron microscopical appearance of the tissue (Fig 1 c) shows all the features classified by Hull (1966) as typical of mature brown adipocytes It follows that the harp- and the hooded seals possess functional brown adipose tissue specifically localized to the venous plexuses

Upon diving the cardiovascular system of the seal is transformed into a heart-brain-lung circuit due to a conspicuous vasoconstriction in almost every tissue except the brain and the heart (Irving 1939) The definite advantage of this redistribution of the blood is that the total oxygen store of blood and lungs is reserved for those tissues which are easily damaged by hypoxia *i e* the brain and the heart The obvious disadvantage of this rearrangement, however is the dramatic decrease in heat production which follows the change from aerobic to anaerobic metabolism in the tissues deprived of circulation The cooling incurred by this



a



b



c

Fig. 1 *a* The venous plexus on the pericardium of a harp seal as visualized by angiography during a dive (from Hol *et al* 1974) *b* Respiratory pattern of isolated mitochondria prepared from pericardial brown adipose tissue of a 14 day-old harp seal pup according to Gra *et al* (1970) The oxygen uptake was measured by means of a Clark electrode The reaction mixtures contained the following final concentrations of components in a total olume of 1 5 ml 135 mM sucrose 40 mM N 2 hydroxyethylpiperazine N ? ethane sulphonic acid (HEPES)—buffer pH 7 2 2 mM EDTA 1 mM $MgCl$ 3 3 μM rotenone and mitochondria (0 5 mg mitochondrial protein ml ¹) At the points indicated 10 mM succinate (Succ) 67 μM ADP 1 / g oligomycin and 3 μM (trace A) or 50 μM (trace B) carbonyl cyanide p-trifluoromet h-oxyphenyl hydrazone (FCCP) were added at a temperature of 5 C The numbers abo e each trace represent the specif c respiratory rates (ng atoms oxygen min ¹ mg m tochondr l protein) In trace B the medium was supplemented with 1 bov ne serum albumin These traces demonstrate that the d stinctive property of loosecoupl ng (*i e* resp ratory rate independent of the prese ce of phosphate acceptor) in brown adipose t ssue mitochondria can be o ercome *in r tro* by fortifying the medium with s rumalbumin *b* Electron micrograph of brown adipose tissue from the pericardial plexus region of a young harp seal pup A blood vessel is seen adjacent to a brown ad pocyte Note the large o centration of mitochondria adjacent to fat droplets *I r t* Typical laminar mitochondria from the same section

defecit in heat production is further reinforced by a maintained or increased blood flow to the poorly insulated brain (Irving 1938) Consequently a marked decrease in deep body temperature is observed in the diving seal (Scholander *et al* 1942) in spite of increased insulation due to the peripheral vasoconstriction During such circumstances of negative heat balance warming of the venous return would be of obvious advantage in minimizing cooling of the central body core

Hol *et al* (1975) have demonstrated that the venous return is shunted through the venous plexuses during the dive and the present study has focussed attention on the fact that these plexuses are embedded in functional brown adipose tissue In our opinion this brown adipose tissue–venous plexus complex may function as a high efficiency tubular heat exchange organ when activated Utilization of the thermogenic potential of the brown adipose tissue would presumably be effected by reflex activation of its sympathetic innervation (Smith and Horwitz 1969)

We thank Professor E. Dahl for performing the electron microscopy This work was supported by the Norwegian Council for Science and the Humanities and by the Norwegian Sealing Council

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An Attempt to Estimate the *in vivo* Concentrations of Noradrenaline and ATP in Sympathetic Large Dense Core Nerve Vesicles

By

Hugo Lagercrantz Gabriel Fried and Ingrid Dahlin

The sympathetic nerve vesicles have generally been assumed to store catecholamines (CA) in the same concentrations and by the same mechanisms as the chromaffin granules from the adrenal medulla Actually some calculations of the number of vesicles in the nerve terminals have been based on this assumption (Dahlstrom Haggendal and Hokfelt 1966) The finding of a molar CA/ATP ratio close to 4/1 in nerve vesicle preparations (Schumann 1958) *i e* about the same as in chromaffin granules was thus expected

However it was surprising to find considerable lower CA content in splenic nerve vesicle preparations apparently consisting to a large part of noradrenaline (NA) storage particles (see Lagercrantz 1971) The low NA concentration in the nerve vesicles from the axons uld be due to immaturity and that the vesicles reach their final NA content on arrival to the nerve terminals This was suggested by Euler (1958) and Stjärne (1968) and further demonstrated by De Potter Chubb and De Schaepdryver (1972) Klein (1973) and Lagercrantz, Kirksey and Klein (1974) Furthermore the finding of low NA contents in the splenic nerve vesicle preparations was to some extent due to NA losses during the critical *post mortem* delay defined as the interval from the time the animals were killed until the nerves could be chilled on ice (Yen Klein and Chen Yen 1973)

The NA/ATP ratio was found to be higher than 4/1 in nerve vesicle preparations when the degree of mitochondrial contamination was considered (De Potter Smith and De Schaepdryver 1970 Lagercrantz and Stjarne 1974) This ratio must also be assumed to be dependent on NA loss during the *post mortem* delay and on whether the nerve vesicles are from the proximal or distal part of the nerve

The aim of the present report is to calculate the true NA and ATP concentrations in the large dense core vesicles of bovine splenic nerve axons *in situ* and also to try to estimate the concentrations in vesicles in the nerve terminals

Bovine splenic nerves were chilled on ice in 0 min (15 ?5) after killing the animals Each nerve was divided in one extra and one intrasplenic section and NA vesicles were isolated from these two sections by differential and density gradient centrifugation on sucrose D_2O gradients as described earlier (Lagercrantz 1971 Yen *et al* 1973) The nerve vesicle fraction obtained in the gradients (fraction III) was diluted to isotonicity with hypotonic suc olution and centrifuged at ?0 000 g_m for 10 min to remove most of the mitochondrial contamination (Lagercrantz and Stjärne 1974) The supernatant containing most of the

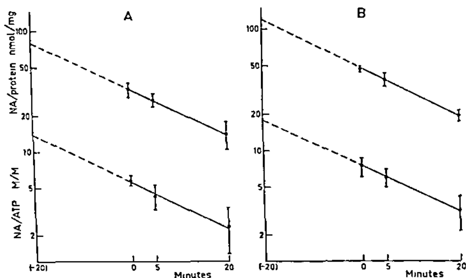


Fig. 1 NA/protein (nmol mg) and NA/ATP (M/M) ratios in NA vesicle preparat ons from extra (A) and intrasplen c (B) nerve segments after incubation at 30 C for 0 5 and 0 min Means of at least 4 expts ±S E. The values are plotted on a semilogaritmic scale with the time of incubation on the abscissa The duration betw en killing the animals and chill ng the nerves on ice is also ind cated on the abscissa (− 0 min) and the ratios are extrapolated back to that moment (dashed lines)

nerve ves cles was incubated at 30 C in the presence of 20 mM K phosphate buffer pH 7 3 and 10 μg/ml Pargyline but without any addit on of NA ATP and Mg^{++} Al quots were removed at the beginn ng and after 5 and 0 min during the incubation and spun down at ∠30 000 g_{max} for 30 min

Analyses (for details see Lagercrantz 1971) The pellets were extracted in 1 ml of 0 4 M perchlori acid for 10 min then 1 ml of distilled water was added After centrifugation 1 ml of the supernatant was used for determination of NA by the trihydroxyindole method and the rest of the supernatant was neutralized to pH 7 0–7 4 with KOH and Tris-buffer The perchlorate precip tate was carefully remo ed by centrif ga tion before storage in the freezer The ATP assay was performed by a modified fire fly method Various amounts of ATP were added to some samples as internal standards The recovery of ATP was on a erage 65 per cent The ATP values were corrected for the losses The first obtained pellet was used for protein assay by the Fol n method

The results are shown in Fig 1 The NA/protein ratio was found to be 32 6 ±2 4 (n = 7 S E) nmol/mg protein in the proximal nerve segment preparation while 45 8 ±2 4 (n = 7) in the distal one (p < 0 01) The NA/ATP ratio was found to be 5 7 ±0 5 mol/mol (n = 10) in the proximal segment preparation and 7 4 ±1 2 (n = 8) in the distal one (p < 0 05) The NA/protein ratio decreased to 50 per cent in about 16 min and the NA/ATP ratio in about the same time About 15 per cent of the ATP content and 16 per cent of the proteins were released during the incubation (20 min)

In order to get an idea of the *in vivo* concentrations of NA and ATP extrapolation was performed from the points obtained at different time intervals during the incubation to the estimated zero time when the animals were killed The release rate of NA and ATP during the *post mortem* delay at the slaughter house was found to correspond fairly well with the release rate during incubation at 30 C (to be published) This was also tested by leaving the nerves at the warm spleens for 20 min before they were chilled on ice (Yen *et al* 1973)

ATP was found to be released slowly compared with NA in accordance with earlier observations (Euler Lishajko and Stjarne 1963 and Stjarne 1964) and not at all released with NA in the stoichiometric ratio 1/4 as from the isolated chromaffin granules (Stjarne 1964) The NA/ATP ratio is calculated to be about 12 in the proximal nerve vesicles and 18–19 in the distal ones

The NA/protein and NA/ATP ratios in the nerve terminals vesicles cannot be determined directly but by comparison with the increase of the NA/dopamine β-hydroxylase ratio in vesicle preparation from the proximal nerve segment and a crude vesicle preparation from the spleen the NA/protein ratio can be assumed to be 400–500 nmol/mg and the NA/ATP ratio about 50/1 (*cf* Lagercrantz *et al* 1974)

Conclusions The *in vivo* amine concentration expressed as nmol NA/mg protein was estimated to be 80 120 and 400–500 in the large dense core nerve vesicles from the distal and proximal segment and the spleen respectively The NA/ATP ratios (M/M) were estimated to be 12/1 18–19/1 and in the range of about 50/1 in the corresponding nerve vesicle preparations

This study was supported by Magnus Bergvalls Stiftelse and Stiftelsen Lars Hiertas Minne

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Dual Adrenoceptor-mediated Control of Noradrenaline Secretion from Human Vasoconstrictor Nerves Facilitation by β-receptors and Inhibition by α-receptors

By

LENNART STJARNE AND JAN BRUNDIN

Previous work with isolated superfused strips of human blood vessels has provided evidence that vasoconstrictor nerves in man possess neural α adrenoceptors capable of mediating negative feedback control of the secretion of noradrenaline (NA) (Stjarne and Gripe 1973) The present study was prompted by reports based on work with different tissues and species that sympathetic nerves may in addition posses β-adrenoceptors enhancing NA secretion and thus capable of mediating positive feedback control of this process (Langer *et al* 1974 Stjarne 1975 a) The aim of the present work was to examine the possible role of β-adrenoceptors in the control of the secretion of NA from human vasoconstrictor nerves

Isolated strips of omental arteries and veins obtained from 8 female patients aged 26 to 95 years and undergoing abdominal surgery under various forms of routine general anesthesia were used for the experiments Premedication always included atropine 0 5 mg s c

The methods used for setting up superfused field stimulated strips of arteries and veins have been explained in detail elsewhere (Stjarne and Gripe 1973 Stjarne and Brundin 1975) The tissues were preincubated with 10 / Ci (260 ng) H-(−)-NA (New England Nuclear Corp) and 0 6 μM desipramine and 10 μM normetanephrine were added to the superfusion medium (Tyrode solution aerated with 6 5 ₒ CO in O_2 temperature was 31 C and flow rate 2 5 ml min^{-1}) to prevent rebinding of NA The secretion of NA evoked by field stimulation of the vasoconstrictor nerves with trains of 300 shocks at 1 Hz (biphasic pul es 1 5 ms in duration applied voltage on Grass S44 stimulator 90 V) was monitored by measuring Δt the fractional secretion of H NA per applied shock (Stjärne and Gripe 1973 Stjarne and Brundin 1975) The value for Δt in each experiment was normalized in relation to the average obtained during the first two control stimulations in that same experiment which was given the value of 1 relative unit

The obtained value for Δt per shock during the initial control period was (4 09 ± 0 21) 10^{-4} (n = 40) which is close to previous observations (Stjarne and Gripe 1973 Stjarne and Brundin 1975)

In agreement with the above mentioned finding in isolated cat and guinea pig tissues

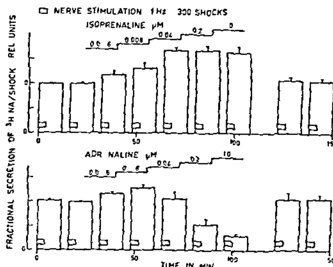


Fig. 1. Fractional secretion of ³H NA per shock (Δt) from strips of human omental arteries and veins (pooled data, relative units, cf. Text). Effects of isoprenaline (4 expts.) and of adrenaline (7 expts.). Means ± S.E.

(Langer *et al.* 1974; Stjärne 1975 a) isoprenaline (L-Isoproterenol-D-bitartrate, Sigma) enhanced Δt ($p < 0.001$). The effect was seen already at 0.0016 µM, and increased with the concentration to reach its maximum, a rise of 67.7 ± 6.2% ($n = 4$), at 0.04 µm (Fig. 1). This shows that human vasoconstrictor nerves possess extremely sensitive β-adrenoceptors capable of markedly enhancing the amount of NA secreted per applied shock.

According to Langer *et al.* (1974) the secretion of NA from sympathetic nerves in cat and guinea pig tissues is depressed by the β-antagonist propranolol 0.1 µM. Their interpretation was that the secretion of NA from sympathetic nerves is in part dependent on β-adrenoceptor mediated positive feedback control.

However, propranolol 0.1–1 µM had no marked effect on Δt in the present study. β-adrenoceptor mediated positive feedback mechanisms thus do not seem to be indispensable or even *quantitatively important for the basic processes of NA secretion from* human vasoconstrictor nerves. Some clue as to the possible physiological function of the extremely sensitive neural β-adrenoceptors may be obtained from the finding that (−)-adrenaline (A), at concentrations known to occur in peripheral plasma in man during enhanced *adrenomedullary secretion (Vendsalu 1960), significantly ($p < 0.001$) enhanced the secretion of ³H NA (Fig. 1). At 0.0016 µM the rise was 15.0 ± 3.0% ($n = 7$) and at 0.008 µM 26.2 ± 7.7%* ($n = 7$). At higher concentrations A depressed Δt, presumably *via* the less sensitive α-adrenoceptor mediated control mechanism (Stjärne and Gripe 1973).

The present findings show that the secretion of NA from human vasoconstrictor nerves may be controlled by a dual set of adrenoceptors with antagonistic effects on the secretory process. The low sensitivity of the α-adrenoceptors suggests that they can only be triggered by NA at the high concentration occurring in synaptic clefts, and that their rôle is to exert negative feedback control of NA secretion. The extremely high sensitivity of the β-adrenoceptors which should enable them to detect circulating catecholamines suggests that they may subserve the function of enhancing the secretion of sympathetic neurotransmitter during conditions of increased secretion of adreno-medullary hormone (Stjärne 1975 b).

This work was supported by grants (to L. S.) from the Swedish Medical Research Council (project B73 04X 3077-05) and from Magnus Bergvalls Stiftelse. We thank Mrs Eivor Stjärne, Mrs Ing Marie Eriksson and Miss Linda Karlsson for skilful technical assistance.

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Acta physiol scand 1975 94 142–144
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Effects of 7-Hydroxy-Δ^6-Tetrahydrocannabinol and Some Related Cannabinoids on the Guinea Pig Isolated Ileum

By

SUNE ROSELL AND STIG AGURELL

Since the structure of Δ^1 Tetrahydrocannabinol (Δ^1 THC) was elucidated by Gaoni and Mechoulam (1964) some 15 other cannabinoids have been isolated Both Δ^1 THC and monohydroxylated metabolites are found in mouse brain following intravenous injection of Δ^1 THC (*cf* Jones *et al* 1974) and it has been suggested that the CNS activity of Δ^1 THC is mediated wholly or in part by the monohydroxylated compounds (Grunfeld and Edery 1969 Ben Zvi Mechoulam and Burstein 1970 Mechoulam 1970 Christensen *et al* 1971 Lemberger *et al* 1972 1973) Δ THC is about as active as Δ^1 THC (Edery *et al* 1972) and appears to have similar psychological effects in man (Hollister and Gillespie 1973)

To investigate the mechanisms of action exerted by the psychoactive cannabinoids isolated peripheral tissues may serve as test objects provided that there is a close relationship between the behavioural actions and the effects on the isolated organ In addition the cannabinoids should be active in concentrations which produce the behavioural effects The present investigation indicates that the isolated guinea pig ileum may be suitable as such a test object

We have studied the effects of 4 cannabinoids Δ^6 tetrahydrocannabinol (Δ^6 THC) 7 hydroxy Δ^6 tetrahydrocannabinol (7 OH Δ^6 THC) cannabidiol (CBD) and cannabinol (CBN) on the guinea pig isolated ileum The substances were dissol ed in ethanol wh ch in the concentrations used had no effects The terminal portion was used after the 5 cm nearest to the ileo-caecal junction had been discarded The *isolated ileum was suspended in Krebs solutio ı (5 ml) bubbled with 95 O and 5 CO The temperature* was 36–37 C Rectangular electrical pulses of 0 5 ms duration supramaximal strength (50 V) and 0 1 Hz were applied to the electrodes Isometric or isotonic contractions were recorded by means of transdu ers (Grass FT 03 and Harvard Apparatus respecti ely) and displayed on a Rika Denki ink writer

7 OH Δ THC reduced the twitch response at a concentration in the bath of approximately 1 ng/ml or higher (*Fig* 1) The effect was noted within 2 min and was maximal within 10–15 min Despite repeated washings the twitch inhibition persisted for about an hour following 1–5 ng/ml On account of the high lipid solubility of the cannabinoids this may not be unexpected Due to the long duration of action it was difficult to establish an accurate threshold dose and dose response curves In some experiments we stimulated the ileum with acetylcholine histamine or 5 hydroxytryptamine (5 HT) 7 OH Δ THC reduced the

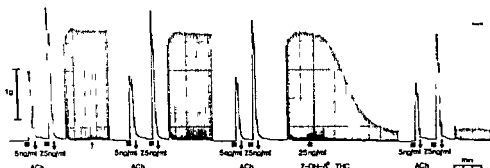


Fig 1 Effect of 7 OH Δ THC 25 ng/ml on twitches and acetylcholine responses of guinea pig il um Acetylcholin was added (■) to the bath wh le st mulat on was temporarily stopped Washings indicated by ↓

twitches evoked by 5 HT but those evoked by acetylcholine or histamine were not at all or very little affected (Fig 1) These data indicate that the inhibition of the twitch is of presynaptic origin Both histamine and acetylcholine act directly on the smooth muscle whereas 5 HT primarily acts by stimulating the nervous structures of the intestinal wall (Rocha e Silva Valle and Picarelli 1953)

Δ¹ THC also inhibited the electrically induced twitches but was approximately 10 times less potent than 7 OH Δ THC Furthermore the inhibition was much more gradual in onset (Fig 2) CBD and CBN were without effect at concentrations up to 100 ng/ml as also was found by Layman and Milton 1971)

The quantitative contribution of the 7 hydroxylated metabolites of Δ¹ and Δ¹ THC towards the psychopharmacological activity of THC in man is still unresolved However the 7 hydroxylated compounds are more or at least as active as their non hydroxylated analogues (*cf* Gill Jones and Lawrence 1973 Jones *et al* 1974 Brande and Szara 1975)

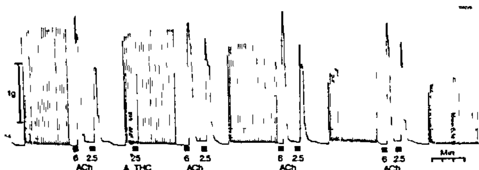


Fig 2 Effect of Δ THC 25 ng ml on tw tches and acetylchol e responses of gu ea pig ileum The numbers nd cate co centrat ons in the bath in ng/ml

CBD and CBN are thought to be much less active (Edery 1972) This order of potency is in agreement with the present results In addition 7 OH Δ⁹ THC is effective on the guinea pig ileum in very low concentrations comparable to those that may be achieved while smoking cannabis Thus cannabis smokers may absorb 1–10 mg of Δ¹ THC to achieve psychic effects (Brande and Szara 1975) If this amount is evenly distributed in the whole body the concentration of Δ¹ THC and its metabolites would be in the order of 50 ng/ml Thus the isolated guinea pig ileum is affected by 7 OH Δ⁹ THC in concentrations which may have psychoactive effects in the human

This investigation was supported by the Swedish Medical Research Council (no 3518 2724) Lotta Malmstrom and Ivan Sandell are gratefully acknowledged for technical assistance

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Acta physiol scand 1975 94 145–153

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Regional and Single Glomerular Blood Flow in the Rat Kidney Prepared for Micropuncture A Methodological Study

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Received 25 October 1974

Abstract

KALLSKOG Ö L. O LINDBOM H R ULFENDAHL and M WOLGAST *Regional and single glomerular blood flow in the rat kidney prepared for micropuncture A methodological study* Acta physiol scand 1975 *94* 145–153

Regional renal and s ngl glomerular blood flow was invest gated in rats using 141 Ce and 85-Sr labelled microspheres The kidneys were prepared for micropuncture and also the artery was catheterized with a steel cannula in order to make injections of 133 Xenon and vasoactive substances selectively into one kidney The mi rospheres were first separated by sed mentation into a narrow fraction and then injected as a plasma suspension into the carotid artery the two batches were infused at half an hour intervals S gle glomeruli were sampled from kidney sections with the aid of s l con rubber casts allowing the ide ti- f cation of th glomerul both w th respect to their localisation and the r postglomerular appearance The results showed a relatively high blood flow in the superficial and juxtamedullary glomeruli of abo e 100 nl/min per 100 g rat wh reas in the other glomerul the blood flow was about 70 nl/min per 100 g rat No differences could be observed between the right untouched kidney and the left prepared for micropuncture Furthermore no discrepancies between the results from the two inject ons could be found The data on the total renal blood flow obtained by the 133 Xenon wash-out method agreed well w th the microsphere method

During recent years haemodynamic conditions within the renal microvasculature have been the object of keen interest especially in regard to the renal handling of salt and water Thus the formation of primary urine is governed not only by the hydrostatic and oncotic forces in the glomerular capillaries but also by the glomerular plasma flow (Brenner *et al* 1972 Deen *et al* 1972) The tubular fluid reabsorption is influenced by the same driving forces operative in the peritubular capillary network (Levy and Windhager 1968 Brenner and Berliner 1969 Falchuk *et al* 1971 Persson *et al* 1972) The study on renal tubular handling of salt and water therefore necessitates investigations of the renal circulatory conditions in addition to studies on the tubular transport kinetics For this purpose the microsphere method (as modified by the use of spheres in a narrow size range (Kallskog *et al* 1972)) has been used to study the regional renal cortical as well as the single glomerular blood flow in rats on animals prepared for micropuncture in the usual manner The aim of this investiga tion has been (1) to estimate the normal flow pattern within different glomerular popula

tions and (2) to test the reproducibility by two consecutive injections of microspheres and (3) to compare the results from the right untouched kidney with the left prepared kidney The latter was exposed via a flank incision free dissected and suspended into a lucite cup The left renal artery was then catheterized in order to measure the renal blood flow with the 133 Xe wash-out method and also to permit the administration of vasoactive drugs selectively into one kidney

The glomerular blood flow was calculated as the mean from 50 glomeruli The glomerular populations were not only classified according to their localisation within the renal cortex but also with respect to their postglomerular appearance permitting studies on the true juxtamedullary glomeruli The results indicate a comparatively high glomerular perfusion in both the very superficial and in the juxtamedullary glomeruli Only minor differences in the values from the right control and the left kidney could be observed thereby indicating that the preparation for micropuncture and renal artery catheterization was accompanied with only a slight disturbance at least as suggested by the circulatory variables studied Furthermore there was no significant difference between the results from the first and the second microsphere injection The total renal blood flow calculated from the microsphere method agreed well with those obtained by the 133 Xe wash out method even though the interpretation from the latter technique in terms of regional renal blood flow (compartmental analysis) can be difficult

Material and Methods

All experiments were performed on male Sprague Dawley rats weighing between 246 and 311 g The animals were anaesthetized with Inactin® (Chemical Fabrik Promonta GmbH Hamburg West Germany) intraperitoneally (120 m /kg B W) tracheotomized and placed on a servo-controlled heating pad Both femoral ies were catheteri ed using polyethylene catheters The left femoral artery was used for blood pressure re ordings and the right artery for continuous sampling of blood during the injection of the m crospheres The right carotid artery was catheterized with a relatively thick (1 mm) catheter with the tip placed in the aortic root just above the aortic valves The left kidney was exposed via a flank incision and suspended into a lucite cup For renal artery cannulation a lumbar artery located just opposite to the left renal artery was free-dissected and tied off A steel cannula with an outer diameter of 200 μm and formed appropr ately was then introduced into the lumbar artery and passed across the aorta into the renal artery With this technique no free dissection of the renal artery or the hilus region was necessary No visible change in the kidney surface could be observed during the catheterization After completion of the surgery the rats were allowed to rest for app ox mately one hour

In the present series 141 Ce and 85 Sr (3M Co Sant Paul Minn USA) labelled 15 μm microspheres were used The spheres were f rst subjected to ultrasonic agitation and then separated by a sed mentat on procedure in large glass cylinders (according to Källskog *et al* 1972) Thus the size range was reduced to about half of the original + 5 μm (± S D) 400 000 spheres were used for each injection—the injection t me was ?0 s At the same time the suction pump connected to the femoral artery catheter was running with a sampling rate of 0 6 ml m n The suction was disrupted some 15 s after the completion of the m crosphere injection The second injection of spheres was made 1/2 1 h after the f rst one

The renal vascular network was then filled with a sil cone rubber compound (Microfil Canton B omed Prod Inc Box 2017 Boulder Colorado 8030? USA) ia an aortic catheter (Bankir *et al* 1973) The kidneys were then excised together w th some other organs such as the brain the heart the lungs the stomach the liver the spleen the suprarenal glands pieces of muscular and adipose t ssue etc and weighed The ki neys were macerated for 30 min in 5 N HCl at 40 C embedded in carboxymethyl cellulose gel and frozen to − 0 C With a microtome 00 μm thick sections were cut in the longitudinal d rection of the kidney (see Fig. 1) From these sect ons glomeruli were sampled manually with the guidance of a m croscope w th 0 times magnif cation The glomeruli were subdivided with respect to their localisation ri (a) superf cial glomeruli (type I) (b) midcort cal glomeruli (II) (c) deep glomerul without vasa recta (III a) and (d)

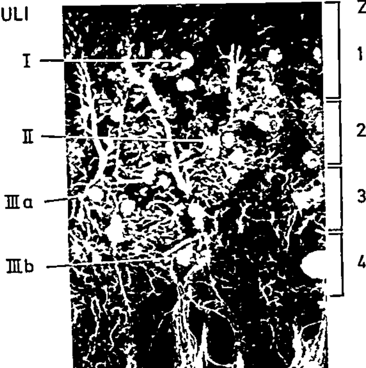


Fig. 1 Microphotograph of a silicon rubber cast showing the subdivision into different zones (to the right) and the different glomerular populations studied (to the left)

glomeruli which by microdissection were found to empty into vasa recta (III b)—the so-called true juxtamedullary glomeruli (see Fig. 1) It should be emphasized that these latter glomeruli mostly were located in the juxtamedullary zone but also not rarely in the middle of the cortical parenchyma At least 50 glomeruli of each type were sampled After the sampling the cortical part of the sections were subdivided into four zones according to Fig. 1 Zone 1 will then constitute the superficial cortex zones 2 and 3 the mid cortical area and zone 4 the juxtamedullary region Effort was made to make the zones representative for the corresponding parts of the whole kidney The blood and tissue samples were analyzed with respect to their 141 Ce and 85 Sr content in a gammaspectrophotometer (Nucab AB P O 039 S-4 1 0- Västra Frolunda Sweden) The total amount of activity injected was determined from the activity obtained from the inject on syringe before and after the injection of the microspheres The blood flow to the specimen was calculated according to $f = M/M_s$ where f is the blood flow f the sampling rate M the activity in the specimen in question and M the activity found in the blood sample Cardiac output (or more correctly the cardiac output minus the coronary blood flow) was determined by the same equation but the factor M will denote the total amount of activity injected and f will represent the cardiac output

In some experiments the renal blood flow was investigated using the 133-Xe wash-out technique in order to make repeated determinations. For this purpose about 10 μCi of 133 Xe in 50 μl of saline was rapidly injected into the renal artery catheter The counting rate was recorded by a gamma detector with the collimator focused over the kidney The blood flow was determined from the slope of the wash-out curve (according to Thorburn *et al* 1963)

Results

The results from the experiments are depicted in Table I Some of the variables are also shown in Fig 2 and 3 The cardiac output was calculated as 32.7 ml/min per 100 g rat decreas

TABLE I Data on systemic and renal circulatory parameters obtained from 2 consecutive microsphere injections The results from the two injections showed no significant difference in any of the variables ($p > 0\ 1$ or more) Likewise there was no difference between the right untouched and the left kidney prepared for micropuncture ($p > 0\ 1$ or more in all instancies)

											Mean ± S D
Rat weight g			246	254	284	284	293	309	311[a]	292	
Perfusion pressure		Contr	113	111	115	109	100	117	130	117	111 7±5 9
mm Hg		Exp	107	107	115	107	96	118	133	115	109 3±7 4
Cardiac output		Contr	45	33	37	32	26	27	39	29	32.7±6 6
ml/min 100 g rat		Exp	40	33	35	29	25	29	38	27	31 1±5 2
RBF left kidney		Contr	2 45	2 14	2 27	2 04	1 53	2 21	2 29	1 80	2 06±0 31
ml/min 100 g rat		Exp	2 56	2 43	2 27	1 78	1 58	2 66	3 19	1 64	2 13±0 45
Blood flow cortical	1	Contr	280 3	142 0	185 6	156 1	81 7	118 9	117 1	114 8	
zones left kidney		Exp	218 8	130 0	207 7	128 5	78 4	149 3	132 2	113 1	
μl/min	2	Contr	154 5	107 1	71 3	124 0	109 5	120 0	164 9	67 5	
		Exp	131 9	144 4	72 5	113 2	107 4	158 7	180 0	67 5	
	3	Contr	122 3	87 9	61 7	93 1	78 3	89 0	78 4	56 4	
		Exp	118 3	133 1	58 4	90 7	80 3	111 2	91 3	59 7	
	4	Contr	108 7	64 5	61 9	61 1	77 5	108 9	138 5	59 7	
		Exp	113 9	110 3	56 6	69 1	85 3	141 2	125 9	72 4	
Glomerular blood flow	I	Contr	154 3	113 6	1 4 3	116 0	62 0	109 7	276 5	178 0	122 6±36 6
left kidney		Exp	119 0	125 8	154 9	103 3	73 4	157 5	175 8	114 3	121 2±29 ?
nl/min 100 g rat	II	Contr	95 2	40 4	77 3	76 1	35 4	96 7	83 5	62 1	68 3±24 2
		Exp	86 5	65 ?	87 2	68 2	55 8	113 2	84 1	85 6	80 2±19 0
	III a	Contr	60 6	86 6	65 4	61 3	59 0	60 9	118 5	61 5	65 1±9 8
		Exp	68 6	95 8	46 9	66 0	44 4	74 9	115 7	52 5	64 ?±18 1
	III b	Contr	102 0	69 0	123 6	111 8	70 4	93 7	166 4	106 4	96 7±20 6
		Exp	98 0	91 0	82 3	125 1	47 4	119 8	143 3	96 0	94 2±25 7
RBF right kidney		Contr	2 89	2 74	1 98	1 80	1 65	2 24	2 73	1 77	2 15±0 49
ml min 100 g rat		Exp	2 94	3 06	1 85	1 73	1 62	2 36	2 52	1 68	2 18±0 61
Blood flow in cortical	1	Contr	165 0	254 9	233 3	138 8	97 8	139 5	68 0	147 6	
zones right kidney		Exp	143 8	230 3	19 1	1 0 1	88 3	163 8	25_ 2	115 5	
μl min	2	Contr	130 7	162 1	99 7	134 9	48 7	109 9	115 4	92 4	
		Exp	97 5	166 4	96 6	1 1 0	57 0	147 9	123 5	99 9	
	3	Contr	72 3	81 9	59 4	75 9	36 1	74 9	82 4	47 4	
		Exp	65 7	109 7	57 3	81 ?	37 1	85 7	84 8	57 0	
	4	Contr	111 _	104 ?	90 6	106 3	38 1	103 6	71 4	_8 4	
		Exp	85 9	133 6	89 7	106 3	45 3	135 9	46 6	39 1	
Glomerular blood flow	I	Contr	137 5	151 0	193 4	116 8	110 3	168 1	259 0	146 9	146 3±28 8
right kidney		Exp	150 4	138 8	148 1	118 9	112 _	174 4	_36 1	109 7	136 2± 37
nl min 100 g rat	II	Contr	74 1	66 3	68 9	76 0	58 1	93 5	125 6	45 0	68 8+15 ?
		Exp	66 0	87 2	46 1	57 0	71 1	96 5	108 9	51 3	67 9±18 6
	III a	Contr	93 5	46 6	45 4	52 8	55 3	62 9	60 0	36 1	54 7± 06
		Exp	75 4	69 4	51 9	56 3	51 3	67 4	16 9	30 9	57 5±14 9
	III b	Contr	106 4	135 6	97 3	123 4	71 4	103	120 0	55 4	99 0±27 9
		Exp	110 9	141 9	69 7	10 20	75 4	112 2	116 1	76 3	98 3±26 1

[a] This experiment is excluded in the statistics

ing insignificantly to 31 1 ml/min in the second injection The scattering of the values was moderate standard deviations were calculated at 7 and 5 ml/min per 100 g rat respectively The left kidney blood flow was 2 1 ± 0 3 ml/min per 100 g rat (mean ± S D) and showed no change during the second microsphere injection Furthermore there was no significant difference between the experimental left kidney and the right untouched kidney the blood flow was almost exactly the same in the two as was the scattering The results on the glomerular blood flow pattern is shown in Table I and also in Fig 2 The blood flow to the superficial glomeruli is then clearly higher than in any other glomerular population amounting to 120

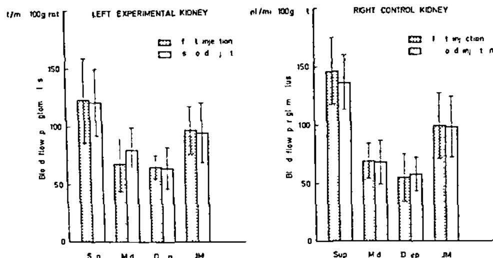


Fig. 2. Glomerular blood flow in nl/min and 100 g rat for superficial (I) m dcortical (II) deep cortical (III a) and true juxtamedullary (III b) glomerul The pattern of distribution with a h gher blood flow in glomeruli types I and III b in relation to types II and III a is highly significant and is val d for both kidneys and for both the first and the second inject on

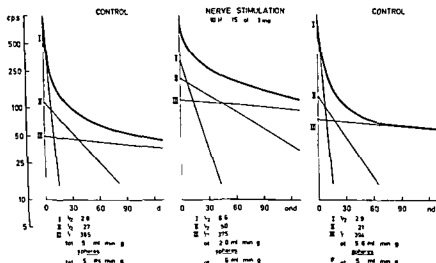


Fig 3 The figure shows the curves obtained from three inject ons of 133 Xenon nto the renal artery catheter of one rat. The f rst curve (to the left) was obta ned during normal control cond tions the second d ring st m lation of th renal nerves and the third again during normal control condit ons The l st jec t on was preceded by two *microsphere inject ons made dur ng control and during renal nerve stimulat on* respecti ely The Xenon wash-out curves ha e been subdi d d to 3 semilogar thm c components The total renal blood flow was calcul ted from the we ghed mean of components I nd II assuming a part t on coeff cient of 0 7 The result ng flow would then refer to more than 90 of the total renal blood flow

TABLE II Student's t test for the comparison of glomerular blood flow rates The table shows that the blood flow to the superficial and juxtamedullary glomeruli (types I and III b) is significantly higher than that to the glomeruli types II and III a The difference between the superficial (I) and the juxtamedullary (III b) glomeruli was barely significant Comparing the midcortical glomeruli (II) with deep cortical glomeruli (III a) showed clearly no significant difference

	Glomerular blood flow first injection						Glomerular blood flow second injection					
	I–II	I III_a	I III_b	II III_a	II–III_b	III_a–III_b	I–II	I–III_a	I–III_b	II–III_a	II–III_b	III_a–III_b
Left kidney	<0 01	<0 01	0 1	<0 5	<0 05	<0 01	<0 01	<0 01	<0 1	>0 1	>0 1	<0 05
Right kidney	<0 01	<0 01	<0 01	>0 1	<0 05	<0 01	<0 01	<0 01	<0 02	>0 1	<0 05	<0 01

nl/min per 100 g rat The difference between the left and the right kidney is fairly moderate and furthermore there are only small discrepancies between the results from the first and second injection In the deeper glomeruli (II and III a see above) the blood flow was considerably less of about 70 nl/min per 100 g rat The pattern of high blood flow in the superficial and juxtamedullary glomeruli compared to the midcortical ones is characteristic and highly significant (see Table II)

The results from the slices given in Table I support the conclusions made above with only a slight difference between the first and second injections

Fig 3 gives the curves obtained from a typical experiment with the 133 Xenon wash-out method during control conditions—after renal nerve stimulation and a new control determination The total renal blood flow was calculated as the weighed mean of compartment I and II to which more than 90% of the total renal blood flow is diverted The last component (III) was not utilized not because of the difficulties in determining the blood flow but due to the hazard in estimating the volume of distribution and its translation into a real volume or weight The former will be influenced by the recirculating indicator and the setting of the baseline and the latter by the difficulties in estimating the partition coefficient (see Thorburn *et al* 1963 Ladefoged *et al* 1965) The results from the two microsphere injections carried out just before the last control study by the 133 Xenon injection are also shown in the figure As was the case in all other studies the results from the two methods agreed

Discussion

The prerequisite for the use of the microsphere method in the determination of regional blood flow in an organ is that the spheres will be distributed in exact proportion to the blood flow and that they all will be trapped in the capillary bed This is especially pertinent with respect to the renal circulation where it has been suggested that axial streaming of the red cells and thereby also of the microspheres in the interlobular arteries would leave a cell poor fraction of blood to perfuse the juxtamedullary glomeruli while the superficial structures should be perfused with a cell rich fraction (Pappenheimer and Kinter 1955) The magnitude of this cell separation however seems indeed to be small Ulfendahl (1962) found that the haematocrit value in the superficial "venae stellatae" of the cat kidney was only about

10 per cent larger than that in the central arterial blood This argument does not rule out *the possibility that large microspheres* (with a large amount of radioactivity) could escape trapping in the deeper cortical regions and then by necessity would be diverted to the more superficial structures This possibility was considered by Kallskog *et al* (1972) showing that the blood flow calculated from the amount of radioactivity trapped agreed well with the relative blood flow calculated from the *number* of spheres trapped Furthermore Bankir *et al* (1973) in an extensive study on single glomeruli perfusion in the rabbit kidney were able to show that the size distribution of the spheres trapped into the different glomerular populations was identical with the distibution in the batch injected This is also supported by the observations of McNay and Abe (1970) showing the same distribution for spheres varying in size from 18 to 36 μm The density of the spheres would not play a large role as essentially equal distribution was obtained from ceramic spheres with a weight of 3 g/ml as for 3M microspheres weighing about 1 5 g/ml A different opinion however was reached by Wallin *et al* (1970) on comparing the regional renal cortical flow pattern in dogs with labelled antibodies and labelled microspheres The two methods corroborated in all the cortical regions except for the very superficial parts where the microsphere method in most instancies gave a higher blood flow value This would be compatible with the concept of axial streaming of the more heavy 3M microspheres This phenomenon could at least partly be explained if unseparated spheres were used where the large spheres and aggregates would be trapped in the interlobular arteries However during volume expansion the two methods agreed In the rat Wallin *et al* (1971 b) found the same pattern of distribution for the two methods during antidiuresis whereas during saline expansion a moderate but significant difference could be observed

Besides systematical errors the scatter can be expected to be large in determinations of small blood flows which obviously is a limitation in studying small changes This scatter is to a great part inherent in the analytical method itself the standard deviation is not equal to the square root of the number of counts analysed but rather to the square root of the number of spheres trapped In the present paper the number of spheres totally injected was choosen so that 1 to 2 spheres should be trapped in each glomerulum As about 50 glomeruli from each population were sampled the variability in the blood flow values ought to be as high as 10–15 per cent A further contribution to this scatter is the variability in the size of the microspheres as the amount of radioactivity per sphere is proportional to the weight of the sphere In our experience the original batch will include large spheres as well as aggregates of spheres which then could cause a variability almost invalidating the method In conclusion measurement of bloof flow in small vascular units more or less necessitates the use of spheres in a narrow size range But even so the variability will most likely be about 20 —as found in the present series

The results from this investigation show an almost complete agreement with that obtained by Bankir *et al* (1973) in experiments on rabbits using the same microsphere technique and the same identification of glomeruli with the aid of silicon rubber casts The most striking feature is the higher blood perfusion in the superficial and the juxtamedullary glomeruli These results however are compatible with the study of Kallskog *et al* (1974) in which the superficial single glomerular filtration rate was 15–20 nl/min per 100 g rat on the same strain

of Sprague Dawley rats This figure should be related to an average single glomerular filtration rate of about 12 nl/min per 100 g rat calculated 1) from the total renal filtration and 2) under the assumption that the kidney will contain 30 000 glomeruli It should be noted in this context that these values are somewhat different from other investigations (see Brenner and Daugharty 1972 Schnermann 1972 de Rouffingnac and Bonvalet 1972)

The majority of the glomeruli in the kidney will however belong to groups II and III where the blood flow was estimated at about 70 nl/min per 100 g rat Utilizing the same number as 30 000 glomeruli/kidney the total renal blood flow is calculated at 2 1 ml/min per 100 g rat This is in reasonable agreement with the figures for the total renal blood flow found in the present series As a matter of fact this ought to be expected as essentially no trapping of the spheres was found in other structures than in the glomerular capillaries

Another source of error is that some spheres could escape trapping due to passage in shunt vessels The finding however that the lung activity only amounted to about 1 per cent of the total amount of activity injected speaks against this possibility The possibility that the Microfil injection could wash away the spheres trapped could also be eliminated as samples of the venous effluent during the microfilling showed nil activity

The major findings in the present investigation are (1) There were no differences in the blood flow and the blood flow distribution between the left kidney prepared for micropuncture and the right untouched kidney (2) The two consecutive microsphere injections gave essentially the same result indicating that administration of about 0 5 mill of microspheres did not seriously affect the heamodynamic conditions

As far as the 133 Xe wash-out technique is concerned it can be concluded that this method is usable for the check up of the renal blood flow and being repeatable it will permit the titration of doses of vasoactive substances renal nerve stimulation etc A more precise evaluation of the blood flow in the different kidney regions during different blood flow levels by compartmental analysis turned out to be uncertain

It is furthermore satisfying that the catheter inserted into the renal artery did not have any major influence The preparation will therefore permit the analysis of the effect of vasoactive substances with respect to the circulatory variables as well as to the tubular function as studied with micropuncture techniques

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Acta physiol scand 1975 94 154–166

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Levels of Cyclic AMP and Electrical Events during Inhibition of Contractile Activity in Vascular Smooth Muscle

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Received 10 December 1974

Abstract

LJUNG B O ISAKSSON and B JOHANSSON *Levels of cyclic AMP and electrical events during inhibition of contractile activity in vascular smooth muscle* Acta physiol scand 1975 94 154–166

The possible relationship between changes in electrical and mechanical activity and in tissue levels of cyclic AMP (cAMP) during relaxation of the myogenically active rat portal vein has been elucidated in response to different inhibitory stimuli Isoproterenol 10^{-8} M caused an initial pronounced inhibition of spike discharge and contractions followed by a partial recovery over the 15 min exposure period This inhibitory response was associated with doubling of tissue cAMP after 1 min and a less pronounced increase in later measurements (5 and 15 min) Papaverine 10^{-4} M caused in contrast an inhibition of electrical and mechanical activity which developed gradually over the entire 15 min exposure period The levels of cAMP at 1 5 and 15 min all exceeded the corresponding values obtained with isoproterenol The two drugs in combination gave a biphasic inhibitory response associated with a sustained large increase in the level of cAMP These drug induced inhibitions were thus accompanied by increased levels of cAMP but the different time courses of the electromechanical responses could not be related to corresponding variations in the content of cAMP Hyperosmolality (150 mM sucrose) and mechanical vibrations (100 Hz) elicited differentiated inhibitions of smooth muscle activity without changes in tissue cAMP The mechanical effects elicited by isoproterenol papaverine and hyperosmolality were accompanied by approximately parallel changes in electrical spike discharg It is concluded that increased levels of cAMP are not indispensible for vascular smooth muscle relaxation and that the magnitude of pharmacologically induced inhibitions re not always closely related to the total tissue content of the nucleotide

In vascular smooth muscle of the single unit type as exemplified by the rat portal vein a close correlation in time is found between the electrical spike discharge and the phasic contractile activity (Funaki and Bohr 1964) There is good evidence that the action potential is involved in triggering contraction in this type of smooth muscle and it is also generally agreed that this control of the contractile system by the electrical membrane events is exerted through variations in intracellular ionized calcium Inhibition of spike activity is an obvious mechanism for inducing relaxation of such smooth muscles but it has been shown that their contractile activity can also be influenced by mechanisms unrelated to electrical membrane events (*e g* Evans *et al* 1958 Johansson *et al* 1967 Somlyo and Somlyo 1968)

A new aspect on the control of smooth muscle activity was introduced by Sutherland and Rall in 1960 when they suggested that relaxation in response to catecholamines is mediated by an elevation of cyclic adenosine 3 5 monophosphate (cAMP) The first experimental evidence for a relation between cAMP and smooth muscle relaxation was the observation that the response of rat uterus to isoproterenol was associated with an increased level of tissue cAMP (Butcher *et al* 1965) With regard to the vascular system recent investigations have shown that various drugs which produce relaxation of different vascular smooth muscle preparations increase the tissue content of cAMP (Volicer and Hynie 1971 Triner *et al* 1972 Andersson 1972) No studies of propagating vascular smooth muscle seem to have been performed in which mechanical activity spike discharge and level of cAMP have been related to one another in the course of induced inhibitions of activity In order to elucidate such possible relationships the present experiments on the isolated rat portal vein were performed Changes in electrical membrane activity mechanical force and cAMP level were analyzed when the muscle was exposed to isoproterenol a stimulator of adenyl cyclase activity and papaverine an inhibitor of the phosphodiesterase enzyme (for ref see Bar 1974) In addition the above parameters were studied when the myogenic activity of the portal vein was inhibited by increased extracellular osmolality which reduces electrical membrane activity (see Jonsson 1970) and by mechanical vibrations which seem to interfere directly with generation of force in the contractile apparatus (Ljung and Siverts son 1975) The experimental design thus allows correlation of tissue cAMP levels to the degree of inhibition when different processes in the series of events which determine activity in the vascular muscle are interfered with

Methods

Isolated portal vein preparations from male rats (200–250 g) of the Sprague Dawley strain were used in these experiments The rats were killed by a blow on the neck the portal vein was carefully dissected cut open longitudinally tied at both ends and mounted for recording by one of the following experimental procedures

Recording of electrical and mechanical activity in the portal vein was performed by the sucrose gap technique as described previously (Axelsson *et al* 1967) The preparation was mounted under a passive force of about 4 mN The hepatic end of the vessel was connected to a force-displacement transducer (Grass FT 03) for recording of isometric contractions This active part of the vein was superfused with standard Krebs solution (composition given below) The preparation was permitted an accommodation period of at least 1 h before the actual experiment started During this accommodation period the muscle was exposed for 5 min to phenoxybenzamine 10^{-6} M which produces irreversible α receptor blockade in the portal vein Four different test solutions were used and these were prepared by adding (1) isoproterenol (10^{-6} M) (2) papaverine (10^{-4} M) (3) isoproterenol (10^{-6} M) and papaverine (10^{-4} M) in combination and (4) sucrose (150 mM) to the standard Krebs The test solutions could be switched in by means of a stopcock system Transient changes of temperature in connection with shifting of the superfusion media were prevented by draining the dead space between the respective aeration chamber and the stopcock immediately before switching The fluid volume between the stopcock and the sucrose gap system implied some dealy in the application of the test solutions this delay has been accounted for in the illustrations below

A total of 8 sucrose-gap experiments were performed The order of application of the test solutions was varied but the time of exposure was always 15 min for each and recovery between exposures was 30 min or more In the following presentation of the results, emphasis will be placed on the changes in phasic electrical and mechanical activity whereas changes in the level of the resting membrane potential will be judged with great caution since they were determined by the sucrose gap method Small variations in super

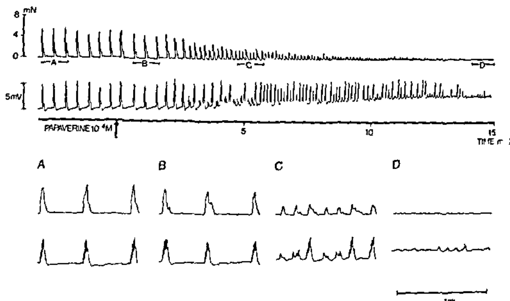


Fig 2 Isometric contractions (upper tracing) and electrical membrane activity (lower tracing) in the rat portal vein Upper panel illustrates control activity and changes during the 15 min following administration of papaverine (10^{-4} M) at arrow Lower panel shows sections of same recordings on an expanded time scale

the papaverine response is assumed The time course of the response in Fig 3 therefore seems to represent the superimposed time courses of the responses illustrated in Fig 1 and 2 an immediate inhibition due to isoproterenol a temporary recovery related to the dwindling isoproterenol effect and the delayed onset of the papaverine effect and finally the gradual decline of activity due to papaverine

Hyperosmolality as illustrated by Fig 4 produces an inhibitory response quite different from those caused by isoproterenol or papaverine The frequency of burst discharges and phasic contractions decreases markedly whereas the spike activity within the bursts and the contraction amplitude are relatively well maintained The decrease in integrated contractile activity is most pronounced in the early phase of the response but is evident throughout the exposure period The level of the resting membrane potential did not change consistently in the different experiments with hyperosmolality This inconsistency was probably due to changes in flow rate of the solution as pointed out above

The inhibitory effect on the spontaneous activity of longitudinal vibrations applied to the portal vein at a frequency of 100 Hz and ± 250 μm in amplitude is illustrated in Fig 5 For technical reasons the electrical membrane activity was not studied in these experiments A small elevation of the line of passive force is seen during the 15 min vibration period The contraction amplitude becomes reduced to one fourth of the control value in response to vibrations but the frequency of contractions remains unchanged After the vibrations the spontaneous contractions regains the same amplitude as in the control situation The imposed length changes thus reduces the isometric force developed by the smooth muscle in a stable and reversible way without affecting the temporal pattern of activity

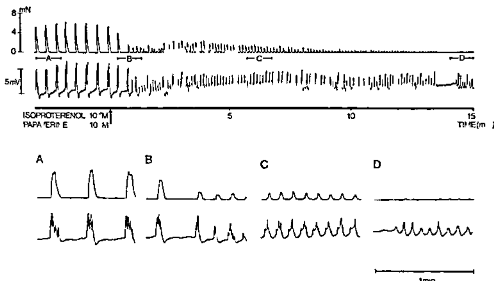


Fig 3 Isometric contractions (upper tracing) and electrical membrane activity (lower tracing) in the rat portal vein Upper panel illustrates control activity and changes during the 15 min following administration of isoproterenol (10^{-5} M) and papaverine (10^{-4} M) in combination at arrow Lower panel shows sections of same recordings on an expanded time scale

The effects of the different inhibitory influences on the cAMP content and on the mechanical activity of the rat portal vein are summarized in Fig 6 and 7 The cAMP level of the smooth muscle in the control situation was determined in 8 preparations which were frozen after the spontaneous activity had been quantitated and found stable during a 20 min period The value obtained 10 8 ± 0 5 pmol/mg protein (mean ± S E) is indicated as the basal cAMP level in the figures Administration of isoproterenol 10^{-} M (Fig 6 A) decreased the mechanical activity to an average of 30 per cent of control during the first min but a gradual recovery occurred during the 15 min exposure period The cAMP level reached a peak value during the first min (19 4 ± 3 8 pmol/mg protein) and remained elevated throughout the 15 min period of isoproterenol influence

In contrast papaverine (10^{-} M) caused a gradual decrease in mechanical activity so that 80 per cent remained during the first few min after administration of the drug whereas practically all activity vanished after a 10 min exposure period The cAMP level was already during the first min considerably increased and reached 31 5 ± 5 5 pmol/mg protein in the 5 min samples

When isoproterenol (10^{-4} M) and papaverine (10^{-} M) were administered in combination (Fig 6 C) a biphasic inhibitory mechanical response was obtained (*cf* Fig 3) The concomitant increase in cAMP attained 29 9 ± 7 4 pmol/mg protein during the first min of exposure to the drugs in combination the peak value at 5 min amounted to 45 6 ± 8 3 pmol/mg protein

In summary the increase in cAMP in response to isoproterenol was temporally correlated to the reduction in contractile activity Papaverine caused considerable elevations in

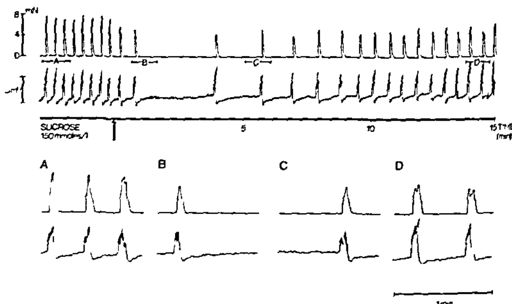


Fig. 4 Isometric contractions (upper tracing) and electrical membrane activity (lower tracing) in the rat portal vein Upper panel illustrates control activity and changes during the 15 min following administration of hyperosmotic solution (+150 mM sucrose) at arrow Lower panel shows sections of same recordings on an expanded time scal

cAMP content within 1 min after administration of the drug, but no significant mechanical response was seen until after 3 min

When the osmolality of the bath solution was increased by addition of sucrose 150 mM (Fig. 7 A) there was a pronounced decrease in the averaged contractile force which was sustained for the 15 min exposure period Similarly longitudinal vibrations applied to the smooth muscle reduced the mechanical activity to 20–30 per cent of the control value during the entire 15 min period (Fig. 7 B) Neither the increased osmolality nor the induced vibrations caused any significant change in the level of cAMP

Discussion

The present results confirm earlier observations concerning the close temporal relation between spike discharge and phasic contractions in portal vein (Funaki and Bohr 1964 Axelsson *et al* 1967) It is also evident from the present study that this electromechanical cor

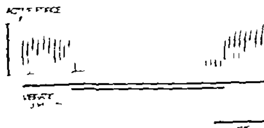


Fig. 5 Inhibitory effect of longitudinal vibrations (100 Hz ...50 μm) on the spontaneous activity of the rat portal vein

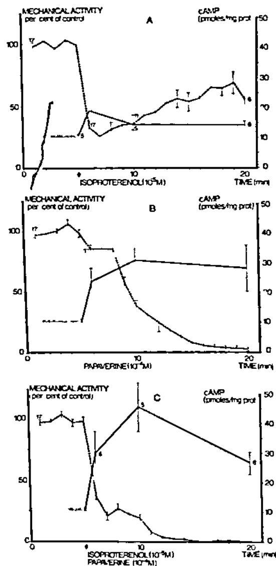


Fig. 6 Mechanical activity and cAMP levels in the rat portal vein exposed to isoproterenol 10^{-8} M (A) papaverine 10^{-4} M (B) or the two agents in combination (C) Mechanical activity expressed as a percentage of the averaged force developed during the 5 min control period Shaded line indicates cAMP level in eight control preparations Subsequent cAMP values (mean ± S E.) obtained from preparations frozen 1 5 or 15 min after administration of the drugs Values of mechanical activity (mean ± S E) obtained from all preparations studied at each time period Number of observations indicated by figures

relation in time persists during responses to isoproterenol papaverine and hyperosmolality which all produce characteristic differentiated changes in the rhythmicity of the muscle Furthermore the size of the individual contractions during these responses seem to be related to concomitant changes in number and amplitude of spikes within the bursts although the experiments do not yield true quantitative data in this respect The electrical membrane events thus appear intimately involved in the short term regulation of the contractile apparatus in the smooth muscle probably via rapid oscillations in intracellular Ca In analogy with the situation in striated muscle the phasic contraction in portal vein may be

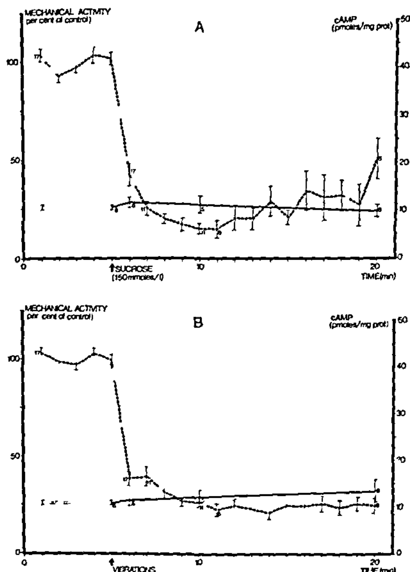


Fig. 7 Mechanical acti ity and cAMP levels (mean ± SE) in the rat portal vein exposed to increased osmolality by addition of sucrose 150 mM (A) and to longitudinal vibrations 100 Hz ± 250 μm (B) Number of observations indicated by figures

triggered by spike activity through influx or release of calcium and relaxation brought about by sequestration of calcium in sarcoplasmic reticulum and mitochondria (Somlyo and Somlyo 1971 Andersson and Nilsson 1972)

In view of the above mentioned maintenance of electromechanical correlation under the influence of isoproterenol papaverine and hyperosmolality it appears that these vasodilator agents exert much of their action through this short term ionic control of the contractile machinery However it is equally clear that the mechanical responses to isoproterenol and

papaverine are accompanied by significant elevations in the tissue content of cAMP This is in agreement with the hypothesis of Sutherland and Rall (1960) that drug induced relaxation of smooth muscle may be dependent on increases in cAMP The present results with hyperosmolality and mechanical vibrations illustrate on the other hand that an increased level of cAMP is not an obligatory requirement for smooth muscle relaxation in general It seems important that the biochemical control of contractile activity which may be exerted by isoproterenol and papaverine via cAMP be reconciled with the changes induced *via* the short term ionic control

Fulfillment of the following two criteria has been emphasized for the establishment of a causal relationship between a change in tissue cAMP and a physiological response 1 changes in the tissue level of cAMP should precede the physiological response and 2 there should be a correlation between the degree of change in cAMP and the magnitude of the physiological response We will first discuss whether the present results from the rat portal vein possibly fulfill these criteria and thus support a causal role of cAMP in this type of vascular smooth muscle

With regard to the effect of isoproterenol the experimental protocol used in the present study does not permit a test of the first requirement due to the fact that inhibition of contractile activity was almost complete before the first measurement of cAMP was made In the case of papaverine the increase in tissue cAMP certainly precedes the marked degrees of relaxation in that an approximately 200 % rise in cAMP was already found after 1 min when only a small relaxant effect could be registered

As to the second of the above criteria some quantitative correlation between the degree of relaxation and the tissue level of cAMP has been obtained in the present study Thus the combination of isoproterenol and papaverine which produced the most pronounced relaxing effect was also associated with the highest tissue levels of cAMP A comparison between the two drugs when applied individually is more difficult Although isoproterenol caused a very rapid and pronounced relaxation the increase in the level of cAMP determined 1 min after addition was small in comparison to the increase obtained with papaverine A typical feature of catecholamine sensitive tissues is however that stimulation of their β-adrenoceptors causes a biphasic increase in the level of cAMP (Dobbs and Robison 1968 Craig *et al* 1969) Therefore the peak level of cAMP in response to isoproterenol might have occurred very early before the first measurement was made in the present study The differences in the time course of the inhibitory mechanical responses obtained with isoproterenol papaverine and the combination of the two drugs are not readily explained by the temporal pattern of cAMP levels found in this study In bovine coronary arteries differences in the rate of onset of relaxation obtained with isoproterenol and papaverine respectively have also been observed by Poch and Kukovetz (1972) However in rabbit colon muscle Andersson (1973) found a temporal correlation between increased cAMP and relaxation induced by papaverine At present there is no obvious explanation for the delayed inhibition of vascular smooth muscle observed with papaverine if this inhibition is to be related to the cAMP level but there are some possibilities which might be considered The phosphodiesterase enzymes found in crude tissue homogenates are active against different purine 3 5 mononucleotides (Beavo *et al* 1970) and it is possible therefore that the level

of cyclic guanosine 3',5'-monophosphate (cGMP) may also be affected by the phosphodiesterase inhibitor papaverine. Information is now accumulating in the literature that different contracting drugs like acetylcholine, carbachol and methacholine increase the level of cGMP in smooth muscle (Schultz *et al.* 1973). Perhaps a transient increase of cGMP in response to papaverine might have counteracted the relaxing effect exerted by this drug via cAMP. Alternatively, the present results might indicate that papaverine initially increases the level of cAMP mainly in a localized compartment of the cell which does not readily affect the contractile system. It has recently been reported (Andersson 1973) that papaverine stimulates cytoplasmic phosphodiesterase activity but inhibits phosphodiesterase activity in the microsomal and mitochondrial fractions of intestinal smooth muscle.

If the present experiments are taken to indicate a causal role of cAMP in the inhibitory actions of isoproterenol and papverine on portal vein despite the above differences in time courses, the mechanisms by which the cyclic nucleotide affects the contractile apparatus must be considered. There is no clear evidence that cAMP is directly involved in the chemomechanical transduction at the level of the actomyosin system. It is more likely that it operates through changes in the ionic control (Ca^{++}) or the supply of energy (ATP) to the contractile machinery. The stimulating action of cAMP on the binding of Ca^{++} to a microsomal fraction of smooth muscles (Andersson and Nilsson 1972, Andersson 1973, Baudouin-Legros and Meyer 1973) is of interest in this connection. However, the present findings indicate that cAMP does not act exclusively by enhancing the removal of cytoplasmic Ca^{++} but also by reducing the supply of Ca^{++} through inhibition of the electrical membrane activity. Earlier work by Somlyo and Somlyo (1969) and by Somlyo, Haeusler and Somlyo (1970) have demonstrated effects of isoproterenol, dibutyryl cAMP and theophylline on membrane potential in vascular smooth muscle and in avian slow striated muscle. Hyperpolarization was obtained with consistency only at low $[K^+]$ (1 mM), whereas relaxation at higher $[K^+]_o$ occurred without concomitant changes in membrane potential or even in association with depolarization. Isoproterenol inhibition of uterine muscle is associated with increased cAMP and hyperpolarization inversely related to $[K^+]_o$ (Kroeger and Marshall 1973). In the present experiments, performed at a $[K^+]_o$ of 5.9 mM, the inhibitory actions of isoproterenol and papaverine always seemed to be accompanied by membrane depolarization (cf. Johansson *et al.* 1967) although the sucrose gap technique does not permit definite conclusions in that respect. It appears that the influence of these drugs on membrane potential may be mediated by an effect of the increased tissue cAMP on ion permeability or electrogenic ion transport (cf. Somlyo and Somlyo 1969, Somlyo *et al.* 1972). Such effects of cAMP could also relate to the changes in phasic electrical activity which are induced by isoproterenol and papaverine, but at present the possible links between these biochemical and electrophysiological events are not known.

As pointed out in the foregoing, the present findings with hyperosmolality show that reduced contractile activity associated with inhibition of electrical activity can occur without variations in cAMP. Under the influence of mechanical vibrations both the level of cAMP and the temporal pattern of muscle rhythmicity remain unchanged indicating that [illegible] of active force in this case may occur directly at the contractile proteins (Ljung [illegible]

son 1975) Also with regard to the responses to isoproternol and papaverine one must in the light of the foregoing discussion seriously consider the possibility that the rise in cAMP is merely an epiphenemon without causal role in the inhibition of mechanical activity

The present experiments have thus illustrated that inhibition of vascular activity may occur through primary influences at different locations in smooth muscle cells In producing this inhibition different patterns of biochemical and electrophysiological alteration have been found

This study was supported by grants from the Swedish Med cal Research Council (3884 4250 14X 8)

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Acta physiol scand 1975 94 167–176
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Release of Gastrin on Vagal Stimulation in the Cat

By

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Received 11 December 1974

Abstract

Uvnäs B K Uvnäs-Wallensten and G Nilsson *Release of gastrin on vagal stimulation in the cat* Acta physiol scand 1975 *94* 167–176

Gastrin released by electrical vagal stimulation was measured in the portal blood of eviscerated cats Blood flow was recorded by a drop chamber technique and the total gastrin output calculated Basal peripheral gastrin levels averaged 65 pg/ml and basal portal levels 225 pg/ml On unilateral vagal stimulation with frequencies above 3–4 Hz gastrin release rapidly increased reaching a peak within 5–10 min In spite of continued stimulation and independent of frequency the gastrin levels declined and returned to basal values after a total of 2–3 000 stimuli A new maximal response could be induced first after a recovery period of 15 30 min In the same cat stimulation of either the left or the right vagus released equal maximal amounts of gastrin (average 32 000 pg) The maximal gastrin output on vagal stimulation corresponds to less than 1 of the total content of antral gastrin determined with radioimmunoassay

The concept of vagal control of gastrin release was originally formulated by Uvnäs (1942) who observed in acute experiments on cats that not only was the gastric acid secretory response to electrical vagal stimulation abolished or considerably reduced by extirpation or cocainization of the antrum but also that this secretory response could be re-established by cross-circulating blood from a vagally stimulated donor animal The validity of the Uvnäs hypothesis has been confirmed by later experiments in which indirect evidence for vagal release of gastrin was obtained in dogs following sham feeding (Pe Thein and Schofield 1959 Olbe 1963) and insulin induced hypoglycemia (Burstall and Schofield 1953) and in cats after reserpinization (Emås and Fyro 1965) and electrical vagal stimulation (Fyro 1967)

Recently the use of radioimmunoassay techniques has allowed the direct demonstration of vagally induced gastrin release in dogs on sham feeding (Nilsson *et al* 1972) electrical vagal stimulation (Lanciault *et al* 1973 Becker *et al* 1974) and insulin hypoglycemia (Jaffe *et al* 1970) Many observers have reported that a release of gastrin—recorded as a rise in the plasma gastrin level—occurs in humans after administering insulin (Ganguli and Elder 1971 Hansky *et al* 1971) However plasma gastrin concentrations alone do not adequately reflect the gastrin output in response to a certain stimulus In the present study we have

attempted to obtain quantitative information concerning gastrin output by recording simultaneously gastric blood flow and gastrin concentrations in the gastric and/or the peripheral venous blood

Methods

Operative procedures

The experiments were performed on 30 cats (2 to 4 kg) fasted for 18 h and anesthetized with chloralose-urethane (50 and 100 mg/kg i v) The lung ventilation was kept constant by artificial respiration and bronchial accumulation of mucous was minimized by continuous suction Blood pressure was recorded in one of the femoral arteries Animals with a blood pressure below 100 mm Hg were excluded from the study Rectal temperature was kept at 38 39 C by heating the animal with an electric heating pad

The intestine below the duodenum was removed To allow sampling of antral gastrin the portal vein was ligated close to its bifurcation (i e above the entrance of the antral venous blood) and a polyethylene catheter was inserted into the blind end of the superior mesenteric vein The remaining retrograde superior mesenteric blood flow containing blood mainly from the stomach (but also blood from the duodenum and the pancreas) was led via the catheter to a recording drop chamber unit and then back into the cat via a cannulated femoral vein (Fig 1) This blood will be referred to as gastric blood In order to keep the antral pH constant a catheter was inserted into the duodenum pushed up into the antrum and fixed in position by a ligature around the pylorus During each experiment the catheter was continuously perfused with warm (38 C) isotonic 0 15 M phosphate buffer solution (pH 7 4) at a rate of 5 ml/min The infused solution was allowed to leave the antrum by overflow through a plastic cannula inserted into the stomach just orally to the antrum-corpus boundary The supine position of the cat and the site of fixation of the gastric cannula ensured a complete filling of the antrum with a small overflow pressure

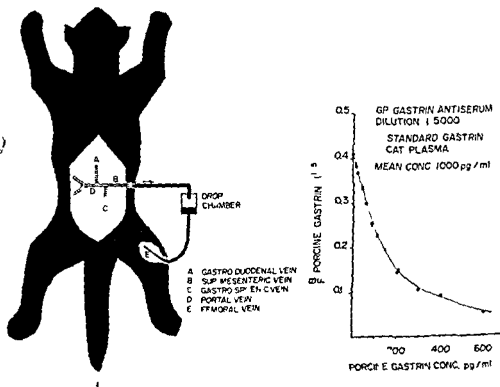


Fig. 1 Schematic drawing illustrating the technique used for recording gastric blood flow and sampling blood for gastrin determination

Fig. 2 Ratio antibody bound (B) to free (F) I[125] porcine gastrin I as a function of the concentration of unlabelled porcine gastrin I and serial dilutions of cat plasma

The cervical vagal nerves were ligated or cut and covered with paraffin oil or Plastibase (E R Squibb & Sons) to prevent drying The distal ends of the nerves were stimulated supra maximally with a Grass stimulator (S 88) delivering stimuli of 7 V 2-5 ms duration and 1-.0 Hz. The effects on heart rate and blood pressure following st mulation were taken as evidence of vagal acti ation

In certain experiments gastric secretion from the gastric cannula was collected each 15 min The volume was measured and the acidity determined by titration with 0 01 M NaOH using phenolphtalein as indicator

Rad oimm noassay

Plasma gastrin concentrations were determined by rad oimmunoassay using labelled porcine gastrin Gastrin antibodies were produced in guinea pigs by injection of crude extracts from porcine antra. The assay technique permitted the detection of 0 8 pg/ml of gastrin in a standard solution Plasma samples were assayed at dilutions of 1 10-1 100 No cat gastrin was available as standard However when tested over a 15 fold concentration range cat plasma gastrin behaved immunochemically as the porcine gastrin standard used (Fig. 2) Further details of the radioimmunoassay have been given elsewhere (Nilsson in press)

1 ml samples of gastric and/or femoral blood were collected repeatedly during the experiments Gastric blood was taken before it entered the drop chamber unit Blood samples were immediately centri fuged at 2 000 rpm for 10 min The plasma was removed, frozen and kept at −20 C until the gastrin assays were performed In order to compensate for blood loss due to the repeated sampling corresponding amounts of physiological saline were given intravenously After completion of all surgical procedures the cats were heparinized to pre ent blood clotting in the catheters Care was taken to ensure that the heparin concentration did not exceed 10 i u /ml

The continuous recording of gastric blood flow allowed quant tative calculations of the gastrin out put to be made Hematocrit values for blood from 10 cats varied between 35-56 —average 47 5 However measurements of the hematocrit during the relevant periods in 10 expts revealed a variation in one and the same animal of less than 10 per cent from the average value In the present study the total gastrin output has been calculated as 65% of the product of gastric blood flow times (×) gastrin plasma con centration The basal gastrin output/min was calculated from 3 5 gastric blood samples taken during 30 min and the concomitant gastric blood flow To obtain the gastrin release due to vagal stimulation the basal gastrin output was subtracted from the total amount of gastrin released into the gastric vein

Results

Basal output of gastrin

(a) *Peripheral venous blood* The basal gastrin levels in the peripheral blood varied consider ably between cats—in 15 cats between 34 and 111 pg/ml (mean 65 pg/ml) (Table I) How

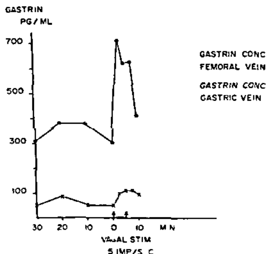


Fig 3 Gastrin in samples of gastric and peri pheral venous blood taken s multaneously The effect of vagal stimulation 5 Hz for 5 min Note the 5 fold d fference in basal gastrin concentrat on between gastric and peripheral blood and the abrupt rise in gastr n level in gastric blood The accompanying rise in the peripheral venous blood is reduced due to the repeated sampling of gastrin rich gastric blood

TABLE I Basal plasma levels of gastrin in (a) peripheral and (b) gastric venous blood. Individual and mean values in 15 cats.

Cat no	Sample no					Mean value
	1	2	3	4	5	
(a) Mean value from all cats 65 pg/ml (34–111)						
1	160	85	75	125		111
2	30	50	40			40
3	60	75	60			65
4	80	80	75	60		74
5	110	110	110	95		106
6	50	75	50	50		56
7	50	40	50			47
8	55	45	40	45	45	46
9	43	55	55	55		5[illegible]
10	50	45	40	45		45
11	37	33	33	33		34
12	50	40	35	40	50	43
13	95	95	75	80	100	89
14	100	100	11[illegible]	100	100	10[illegible]
15	60	65	60			6[illegible]
(b) Mean value from all cats 224 pg/ml (65–605)						
1	580	6[illegible]0	600	6[illegible]0		605
2	150	90	150	150		135
3	180	170	180			177
4	100	75	100	110		96
5	150	150	[illegible]00			167
6	65	90	90			8[illegible]
7	[illegible]00	190	[illegible]00	[illegible]00	210	[illegible]10
8	3[illegible]5	275	350			317
9	140	100	140	160		135
10	180	310	[illegible]10	300	190	[illegible]38
11	75	45	75			65
12	80	80	1[illegible]0	80		90
13	[illegible]00	100	300			[illegible]00
14	310	380	380	310		345
15	400	550	550	550		51[illegible]

ever within experiments gastrin concentrations fluctuated less and were usually within 30% of the mean value

(b) *Gastric venous blood* In general the gastrin concentration in the gastric outflow was found to be about 4–5 times higher than in the peripheral blood (Fig 3) The average gastrin values from 15 cats varied between 65 and 605 pg/ml —mean value 224 pg/ml (Table II) Provided that the gastric blood flow remained constant the basal gastrin levels in the gastric blood showed variations of the same magnitude as in the peripheral blood during the course of an experiment When increases in the basal gastrin concentrations were observed they were usually found to be due to a decrease in gastric blood flow and not to an increase in gastrin output Calculations showed the gastrin output to be unchanged

Vagally induced release of gastrin

Significance of stimulation frequency Adequate vagal stimulation was able to induce an abrupt increase in gastrin release reflected as a rise in the gastrin level not only in the gastric

TABLE II Gastrin release into the gastric vein following unilateral vagal stimulation on 8 cats with ≥2400 impulses

Cat no	Frequency Hz	Duration sec	Number of impulses	Gastrin pg
1	10	4[illegible]0	4 [illegible]00	11 525
2	4	600	2 400	8 [illegible]00
	4	600	2 400	7 600
3	4	900	3 600	52 500
	8	900	7 200	47 213
	15	600	9 000	45 575
4	8	300	2 400	34 188
	8	300	2 400	33 463
5	5	480	2 400	14 850
6	10	300	3 000	32 950
7	8	300	2 400	58 000
8	5	900	4 500	41 200

Mean value from 8 experiments 3[illegible] 000 pg (7 600–58 000)

(Figs 3 and 4) but also in the peripheral venous blood (Fig 3) The stimulation frequency was critical On unilateral stimulation with frequencies below 2-3 Hz no significant rise in the gastrin levels (Fig 4) was observed in the gastric blood Attempts to establish a frequency response curve for the gastrin release met with unexpected difficulties since the release mechanism proved to be rapidly fatigued Within the frequency range studied (4–20 Hz) vagal stimulation induced a rise in the gastrin concentration reaching a peak level

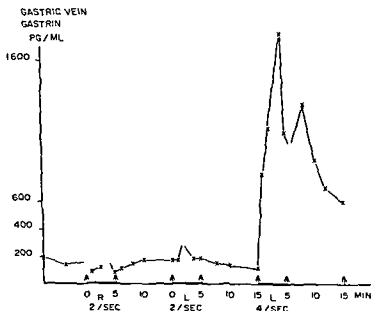


Fig 4 Release of gastrin on unilateral vagal stimulation (4 Hz 5 min) Note that there is no significant release with Hz. R = right, L = left vagal nerve

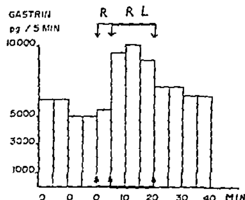


Fig 5 Gastrin release on unilateral and bilateral *vagal stimulation with 2 Hz Note the absence of* response to unilateral stimulation R = right L = left

within 5–10 min Within 10–20 min the gastrin values then declined to near prestimulatory levels regardless of continued stimulation (Figs 6 and 7) The higher the frequency the steeper and higher the gastrin peak and the shorter the release response provided that the blood flow was unchanged

Further analysis of several experiments exhibiting the fatigue phenomenon revealed that within a frequency range of 4–20 Hz (unilat stim) the gastrin release mechanism became temporarily exhausted when the total number of stimuli delivered exceeded 2–3 000 This

Fig. 6 Release of gastrin following vagal stimulation (4 and 8 Hz for 15 min) illustrating fatigue of the release mechanism and subsequent recovery after 15 min Note the decreased gastric blood flow during the second stimulation giving higher gastrin plasma concentrations but unchanged total output R = right

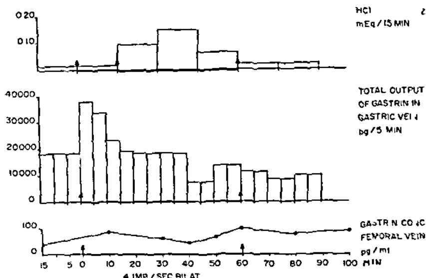


Fig 7 Gastrin output into the gastric vein gastrin concentration in the femoral vein and acid secretory response following bilateral vagal stimulation (4 Hz 60 min) Note the continued gastric acid production in spite of declining gastrin release into the gastric vein

was evidenced by a return of vagally induced gastrin levels to basal as soon as 2–3 000 impulses had been delivered in spite of continued stimulation (Fig 6) The fatigue of the release mechanism occurred independently of frequency or length of stimulation as soon as this critical number of impulses was reached As is evident from Table II the total output of gastrin in response to a supramaximal stimulation varied considerably from animal to animal (7 800–58 000 pg) However within one and the same animal the maximal amounts of releasable gastrin were fairly constant (Table II Fig 6) Stimulations with more than 2–3 000 impulses did not augment the gastrin response (Table II) After depletion 15–20 min were required for full recovery

Stimulation of either the right or the left cervical vagus released gastrin (Figs 4 and 6) Bilateral stimulation increased the total gastrin output and caused a significant rise in the gastric blood with stimulation frequencies as low as 2 Hz (Fig 5)

Importance of concomitant recording of gastric blood flow and gastrin concentration

Initial gastric blood flow varied between 4 and 10 ml/min A slight decline was sometimes seen during the course of the experiments Fig 6 illustrates the importance of simultaneous recording of gastric blood flow and plasma gastrin concentrations for obtaining correct quantitative information about gastrin release In this experiment vagal stimulation with a supramaximal impulse load (3 600 impulses) induced the release of 52 500 pg of gastrin A second vagal stimulation this time with twice as many impulses—7 200—resulted in a more sustained release of gastrin From the concentrations of gastrin one might get the impression that the second gastrin release response (7 200 impulses) was higher than

the first one (3 600 impulses) However since for unknown reasons the gastric blood flow declined during the second stimulation the total gastrin output was in fact lower (47 200 pg compared to 52 500 pg)

Because an antrum buffer perfusion system was used in our studies gastric acid was not collected and measured routinely To find out whether the surgical preparation might have deranged the acid secretory response pattern to vagal stimulation gastric acid was collected and measured in some experiments In these experiments the animal was placed on its left side in such a way that the stomach was continuously drained via a plastic catheter in the gastric cannula As illustrated in Fig 7 vagal activation induced not only the expected temporary rise in the gastrin level in the gastric blood but also a continuous acid secretory response typical of vagal stimulation The quantitative relationship between gastrin output and the acid secretory response will be discussed in a subsequent paper

Discussion

The present results corroborate the 30-year old proposal of Uvnäs (1942) concerning vagal control of the release of gastrin in cats Previously Lanciault *et al* (1973) and Becker *et al* (1973) reported increased gastrin levels in the portal and antral veins respectively on electrical vagal stimulation in dogs Although there is principal agreement between the present results on cats and those previously reported on dogs there are at the same time striking quantitative differences which require some comments

Since cat gastrin was not available the gastrin values obtained with our immunoassay are only relative However our cat plasma gastrin has been shown to behave immunochemically in the same way as the porcine gastrin standard Furthermore the mean peripheral plasma value found in our cats (65 pg/ml) is of the same magnitude as found in other species (*e g* man and dog) These facts indicate that our immunoassay technique is reliable

We observed a 4–5 fold higher basal gastrin concentration in the gastric blood than in the peripheral venous blood The gastrin concentration in the gastric blood increased on vagal stimulation up to 10-fold reaching a peak after only a few minutes The higher the frequency of stimulation and the more rapid the gastric blood flow the quicker the peak of gastrin output appeared At a frequency of 20 Hz, as used by Becker *et al* for example (see below) we observed a peak output already within 30 s

In spite of continued stimulation the gastrin release rapidly fell to reach the prestimulatory level within 10–20 min Such a fatiguability was observed after stimulation with a total of 2 000–3 000 impulses provided the stimulation frequencywas in the range of 4–20 Hz. Full recovery of the release mechanism required a resting period of 15–20 min

In contrast to our results Lanciault *et al* found in their dogs no difference between the basal gastrin levels in the portal and the peripheral venous blood Further they reported a 2–3 fold increase in portal gastrin levels even after 30 min continuous stimulation of the cervical vagi (frequency 5 Hz)

The discrepancies between our results and those of Lanciault *et al* may be due to technical factors They determined gastrin concentrations in portal blood withdrawn from a

catheter inserted into the portal vein No control of the position of the tip of the catheter is mentioned and with such a sampling technique an uncontrollable admixture of blood from non antral sources cannot be avoided In fact their finding of almost equal portal and peripheral prestimulatory gastrin levels indicates that in their dogs antral blood formed only a minor part of the portal blood withdrawn In the absence of simultaneous blood flow measurements their report of a 2–3 fold elevation of the portal gastrin level even after 30 min continuous vagal stimulation is of questionable validity The elevation might equally well be explained as being due to reduced blood flow or less admixture of non antral blood as to vagally induced gastrin release

The pronounced fatiguability of the gastrin release mechanism to vagal stimulation observed in our study may have several explanations A rapidly occurring fatigue of vagal neurotransmission processes cannot be excluded but seems rather unlikely especially in view of the recovery time (15–20 min) which is unreasonably long for cholinergic trans mission fatigue In our view our observations indicate the existence of a small antral pool of gastrin easily depletable by vagal impulses Similarly in the dog the gastrin release mechanism seems to be easily exhausted Electrical vagal stimulation also in this species leads to a transient rise in gastrin output (unpublished observation)

The reason why the existence of this depletable gastrin pool has been overlooked by previous investigators is probably the different sampling techniques used In order to detect and measure quantitatively the transient initial gastrin peak repeated blood sampling is required during the first few minutes of stimulation In the experiments of Lanciault *et al* the initial gastrin peak has almost certainly been missed since the first blood sample was not taken until after 30 min of stimulation

The average size of the depletable gastrin pool was 32 000 pg (see Table II) indicating that only a minor part of the total antral gastrin is immediately available for release by vagal impulses Nilsson *et al* (1973) have determined the total content of gastrin like material in cat antrum to be about 10^7 pg Under the assumption that the immunoreactive material corresponds to gastrin or closely related precursor material less than 1 % of the total gastrin store should be released by one supramaximal vagal stimulation (2 000–3 000 impulses)

The stimulation frequency used by us to demonstrate the fatiguability of the gastrin release mechanism (>4 Hz) is probably unphysiologically high The physiological vagal impulse frequency range is assumed to be around 0 5–4 Hz e g graded acid responses are obtained following vagal stimulation within this frequency range (Sjodin 1975) Also stimulation with such low frequencies suffices to induce and maintain HCl secretion for hours—suggesting a concomitant release of gastrin We did not regularly record the gastric secretory response to vagal stimulation but in those experiments in which the HCl secretion was measured a sustained secretion was observed throughout the experi ments as illustrated in Fig 7

In our experiments unilateral vagal stimulation with frequencies below 4 Hz did only rarely cause a detectable rise in the gastrin output This inability to record any gastrin release at frequencies within the physiological frequency are most certainly due to defi ciencies *in our recording technique As already mentioned under Methods* our gastric blood contains a mixture of venous blood mainly from the antrum and the corpus of the

stomach (but also from the pancreas and the duodenum) the blood from the antrum forming a minor part. Consequently the original gastrin concentrations in the antral blood will be considerably reduced due to the admixture with blood relatively poor in gastrin. Since the basal gastrin values fluctuate about 30%, our present technique will not always allow us to record the low gastrin output which can be expected on unilateral vagal stimulation with frequencies below 4 Hz. Bilateral stimulation increased the gastrin output compared to unilateral stimulation. Thus bilateral stimulation with 2 Hz raised gastrin output appreciably (Fig. 5).

The generous gift of porcine gastrin I by Professor R. A. Gregory is gratefully acknowledged.
This investigation was supported by grants from Juhlins Fond, Karolinska Institutet and from Statens Medicinska Forskningsråd (3571).

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Acta physiol scand 1975 94 177–183
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Reflex Inhibition of the Slowly Adapting Stretch Receptors in the Intact Abdomen of the Crayfish

By

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Received 19 December 1974

Abstract

NJÅ A and L WALLOE. *Reflex inhibition of the slowly adapting stretch receptors in the intact abdomen of the crayfish* Acta physiol scand 1975 94 177–183

The reflex inhibition of abdominal stretch receptors in crayfish was studied by controlled passive flexion of individual abdominal joints. The results are very similar to those obtained by electrical or mechanical stimulation of individual stretch receptors in immobilized abdomens. Reflex effects from posterior to anterior abdominal segments are somewhat stronger than in the opposite direction Flexion of one abdominal joint excites the stretch receptors of that joint and inhibits the stretch receptors in neighbouring abdominal segments. Without reflex inhibition flexion of one abdominal joint excites the stretch receptors in several abdominal segments.

The slowly adapting stretch receptors in the thorax and abdomen of the crayfish have provided unusual opportunities for the investigation of sensory inhibition The histology of the receptors and their efferent innervation has been described in lobster and crayfish (Alexandrowicz 1951 1952, 1967 Florey and Florey 1955) The peripheral cell body attached to a specialized muscle strand, facilitates isolation of functioning receptors and allows adequate activation by stretch applied to the muscle strand application of various solutions stimulation of inhibitory axons, and recording of synaptic potentials and injection of currents with an intracellular microelectrode These technical advantages have made possible detailed studies on the inhibitory synaptic mechanisms and transmitter action of the large accessory neuron which is the main inhibitory axon (Kuffler and Eyzaguirre 1955 Hagiwara Kusano and Saito 1960)

There is one slowly adapting stretch receptor and one large accessory neuron on each side of each abdominal segment When the neural connections between the receptor and the central nervous system are intact activity in one slowly adapting stretch receptor reflexly activates its own large accessory neuron In addition, this receptor activates the large accessory neurons of several other abdominal segments mainly on the same side of the animal (Eckert 1961) The distribution of this large accessory reflex to different abdominal segments and the input-output relations of the various reflex connections have been determined by electrical and mechanical stimulation of receptors in immobilized abdomens

(Eckert 1961 Jansen Njå and Walløe 1970 b Jansen Njå Ormstad and Walløe 1971 a b) Page and Sokolove (1972) recently published a report which emphasized the non symmetrical distribution of the reflex They found considerably stronger reflex connection from posterior to anterior segments than in the opposite direction when receptors were activated by spontaneous or command fibre induced abdominal flexions The asymmetry of the reflex coupling is small during electrical stimulation of the stretch receptor axons (Jansen *et al* 1970 b)

Because of this discrepancy we investigated the input-output relations of the large accessory reflex during controlled passive flexion of individual abdominal joints The pattern of stretch receptor activation thus obtained has several distinctive features (1) Simultaneous and equally strong activation of stretch receptors on both sides of the animal (2) Selective activation of slowly adapting stretch receptors because the rapidly adapting receptors respond only to full range flexions of high velocity (Njå and Walløe 1973) (3) Possible activation of other kinds of receptors with reflex connections to the large accessory neurons To understand the *in situ* performance of the large accessory reflex results which have been obtained by electrical and mechanical stimulation of the receptors of immobilized abdomens should be supplemented by experiments in which the receptors are activated by joint flexion In this report we show that the reflex activation produced by passive joint flexion in intact abdomens is very similar to that produced by direct electrical or mechanical stimulation of the receptors

Methods

The experiments were performed on the isolated abdomen of fresh water crayfish (*Astacus fl riatil s*) The preparations were bathed in oxygenated saline containing (mM) NaCl 205 KCl 5 4 $CaCl_2$ 13 5 MgCl 2 6 Tris 10 titrated to pH 7 2 to 7 4 with maleic acid The temperature of the bath was thermostatically controlled at 12 C The stretch receptors were activated by controlled passi e flexion of the abdominal jo n s (Fig 1) Most of the experimental set up and the technique for flexing the joints ha e been described earlier (Njå and Walløe 1973)

Action potentials were recorded extracellularly with flexible suction electrodes fitted into circular boreholes (Fig. 1) No suction was needed since the rubber coated electrode shaft stuck to the borehole after it had been inserted as far as necessary to obtain good contact between electrode and nerve (branch of the second root inner ating the extensor muscles) Situated close to the joint axis the electrode to-nerve contact was unaffected during the full range of mo ements in the joint The amplifier activity was d splayed on a cathode ray tube and photographed conventionally on moving film Frequencies were counted over 1 s starting 0 5 s after the beginning of the step

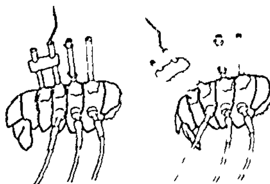


Fig. 1 Typical experimental arrangement 3 simultaneous recordings are made from the right side of the second third and fourth segments Left Zero reference position Right Selecti e flexion of the joint between the third and the fourth segments

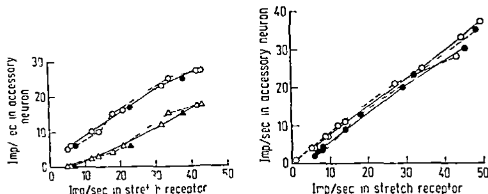


Fig 2 Reflex output from the large accessory neuron as a function of slowly adapting stretch receptor input. The stretch receptor on the right side of the third segment was activat d by pulling the receptor muscle with a fine glass hook (fill d symbols) in between 2 series of flexion experiments (open symbols) C rcles represent the reflex output in the same segment and triangles the reflex output in the next anterior segment. Contralateral stretch receptors were denervated Solid l nes First seri s of fl xion experiments. Dashed l nes Seco d series of flexion e pe ments

Fig 3 Reflex output from the large accessory neuron as a function of bilateral (○) and ipsilateral (●) slowly adapting stretch receptor input The recording was made from the right s de of the second segment dur ng flexion Ipsilateral input was produced by denervation of the receptor on the opposite side Two trials (solid and dashed l nes) were performed in each situat on

In the e periments in wh ch the refle effects of pull ng the rec ptor muscle were compared to those el cited by joint flexion the dorsal part of the tergite of abdominal segments 2, 3 and 4 were removed to expose the stretch receptors b laterally The stretch receptors on the left side of these segments were de nervated The pos t ons of the joints were controlled by means of pins inserted ert cally through the animal on the left side one in each s gment from no 2 through no 5 instead of glass rods in the midline

Results

Input–output relations of the large accessory reflex

Impulse activity in a slowly adapting stretch receptor caused reflex activation of its own large accessory neuron In addition the large accessory neurons of several other abdominal segments were activated Receptor activation by joint flexion produced the same reflex output as that produced by direct stretch of the receptor muscle strand provided that the contralateral stretch receptor had been denervated

The results from one experiment are illustrated in Fig. 2, which shows the ipsilateral reflex outputs from the large accessory neurons in the same and neighbouring segments as a function of impulse frequency in the slowly adapting stretch receptor There was no difference in the reflex output produced either by flexion or by direct mechanical activation of the receptor 5 expts on 4 animals all showed similar results

The effect of bilateral stretch receptor input was compared to that of unilateral input in several experiments Ipsilateral input was obtained after cutting the nerve on the opposite side Fig 3 shows the results from a typical experiment No difference occurred for mo t input frequencies For input frequencies below about 15 imp s however bilateral input was more effective than ipsilateral input

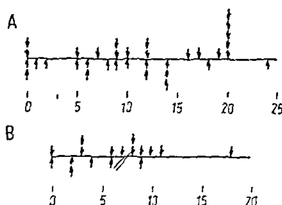


Fig. 4. Efficiency of the large accessory reflex couplings in the anterior and the posterior directions. Data from 17 pairs of large accessory reflexes between neighbouring segments. The input frequency was 20 imp/sec. A. The arrows represent the output frequencies in imp/sec for the reflexes in the anterior (above the line) and the posterior (below the line) directions. B. Similar representation of the differences within pairs (anterior direction — posterior direction). Positive differences are shown above the line.

The distribution of this reflex to different abdominal segments is of obvious functional significance. Page and Sokolove (1972) recently published a report that emphasized the non-symmetrical distribution of the reflex. They found considerably stronger reflex connections from posterior to anterior segments than in the opposite direction and suggested that this could have important consequences in reflex function. We studied the symmetry of reflex coupling in 17 pairs of ipsilateral reflexes between neighbouring segments. In Fig. 4 A the reflex output (in imp/s) evoked by a stretch receptor input frequency of 20 imp/s is shown for each of the 34 reflexes. The difference between the output frequencies in the two directions appears to be small. The point estimate of the median of the output frequencies is 12 imp/s in the anterior direction and 9 imp/s in the posterior direction. The corresponding confidence intervals with a confidence coefficient of 0.95 are 9 imp/s $< \eta <$ 20 imp/s and 5 imp/s $< \eta <$ 14 imp/s (non parametric estimates, see Noether 1971). Fig. 4 B shows the difference in output frequencies within each particular pair of reflexes. In most pairs the output frequency in the anterior direction is greater than that in the posterior direction. The difference, however, is not significant at the 5 per cent level in the present material (α = 0.08 in the Wilcoxon's test for paired comparisons, two-sided test).

Effects of flexing neighbouring joints

Reciprocal inhibition between slowly adapting stretch receptors of neighbouring segments has previously been demonstrated during electrical and mechanical stimulation of the receptors in immobilized abdomens (Eckert 19?1, Jansen *et al.* 1970 a). This mutual inhibitory interaction was also observed during joint flexion in the present series of experiments.

An example is shown in Fig. 5. A slowly adapting stretch receptor was firing at about 5 imp/s after adaption to a constant angle of flexion in its joint. The change in firing frequency in this receptor caused by flexing the next posterior joint was then recorded. Flexing this joint caused a decrease in the firing frequency of the receptor. The inhibitory effect increased with increasing angles of flexion in the next posterior joint and at ?0° the receptor almost stopped firing. Fig. 5 also shows the results obtained when the experiment was repeated on the same receptor after its nerve supply had been cut. In this case flexion of the next posterior joint caused a mechanical excitation of the receptor

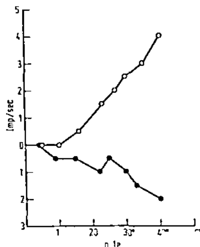


Fig 5 Effect of flexing a neighbouring joint The chan^e in firing frequency of a slowly adapting stretch receptor in the third segment is plotted as a function of the an^le of flexion in the next posterior joint with intact central connections (●) and after nerve section (O) The background firing frequency of the receptor in the third segment was approximately 2 imp/s after adaption to a constant angle of flexion (30°) in its joint

The actual effect of the neural inhibition corresponds to the differe ce b tween the two curves in Fig 5 This difference is considerably greater than the net decrease in impulse frequency evoked by joint flexion (compare with Fig 2 in Jansen et al 1970a) Flexion of the rext anterior joint p oduced s milar results

The excitatory effect of neighbouring joint flexion on slowly adapting stretch receptors was regularly observed after nerve section in these preparations Because it occurred after the receptors had been denervated this excitation appears to be purely mechanical The degree of excitation was different in different preparations but it was usually strong^r in the anterior than in the posterior direction A typical example is shown in Fig 6 Maximal flexion of one joint evoked a firing frequency of about 80 imp/s in its own stretch receptors (without reflex inhibition) and increased the impulse frequency in the receptors of the n^xt anterior segment by about 8 imp/s The excitatory effect was about half this great in the opposite direction Similar but weaker excitatory effects were observed 2 segments away Provided that the steady state background firing frequency in the receptor was above zero the results were largely independent of the valt.e of the constant angle of flexion in its joint

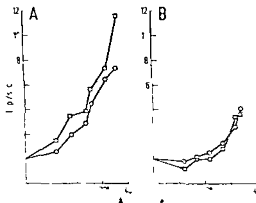


Fig 6 Effect of flex ng the ne ghbourin joints The change in firing frequencies of the denervated right (_) and left (O) slowly adapt stretch receptors of the third seg ment is plotted as a funct on of the angle of flexion in the ne t posterior (A) and the next *anterior (B) jo nt In this preparatio n t al* fl x on of the next anterior joint released the receptors sl ghtly The background f ring fre quenc es of the receptors of the th rd seg ment were a pro mately 5 imp s after adap tat on to a constant angle of fl^xio (30) in th ir joint

Acta physiol scand 1975 94 184–188

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Plasma Renin Activity and *in vitro* Synthesis of Aldosterone by the Adrenal Glands of Rats with Spontaneous, Renal, or Pinealectomy-Induced Hypertension

By

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Received 19 December 1974

Abstract

KARPPANEN H S LAHOVAARA, P MÄNNISTÖ and H VAPAATALO *Plasma renin activity and in vitro synthesis of aldosterone by the adrenal glands of rats with spontaneous renal or pinealectomy induced hypertension* Acta physiol scand 1975 94 184–188

In the present study a comparison was made on the role of the renin aldosterone system in rats with various forms of experimental hypertension (pinealectomy induced, renal and spontaneous) The plasma sodium and potassium concentrations as well as renin activity were measured The *in vitro* production of aldosterone by quartered adrenal glands of these rats was also determined 5 weeks after the operations the blood pressure of the pinealectomized and renal operated rats was significantly increased The plasma sodium concentration did not differ in various groups, but that of potassium was decreased in the renal hypertensive animals The plasma renin activity of the pinealectomized rats was elevated while in other forms of hypertension it was at the control level The basal aldosterone production by the adrenal quarters was equal in all the groups ACTH dibutyryl cyclic adenosine 3 ,5 monophosphate (DBA) and 5HT stimulated the aldosterone production The responses to ACTH and DBA were greater in the adrenals of renal hypertensive rats than in the other forms of hypertension or in the controls We suggest that the renin aldosterone system is of importance in the maintenance of renal hypertension while in pinealectomy induced hypertension elevated plasma renin activity reflects an increased sympathetic activity which probably is the main cause of hypertension in these animals

Key words Experimental hypertension spontaneous hypertension renal hypertension, pinealectomy induced hypertension renin aldosterone system

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In order to elucidate the ethiology and pathogenesis of human arterial hypertension various animal models have been utilized Animals rendered hypertensive by operations affecting the renal arteries or the renal tissue itself have been used for studies of renal hypertension. Okamoto and Aoki (1963) introduced a strain of spontaneously hypertensive rats which have been generally accepted as the best model available for studies of human essential hypertension Recently we have reported that rats with pinealectomy induced hypertension bear some similarity to the spontaneously hypertensive rats (Karppanen 1974)

Despite extensive studies there is no agreement about the significance of the renin antiotensin aldosterone system in the development and maintenance of arterial hyperten sion In the present work we have compared the renin aldosterone mechanism in rats with renal spontaneous or pinealectomy induced hypertension by measuring the plasma renin activity and the *in vitro* production of aldosterone by the adrenal glands of these rats

Materials and Methods

Male Wistar rats 170-180 g, were pinealectomized with the stereotaxic electrocoagulation method previ ously described in detail by us (Karppanen *et al* 1970 Karppanen 1974) The renal hypertension was in duced by removing the right kidney and tightening a figure of-eight ligature around the remaining kidney The spontan ously hypertensive rats were males of the Wistar strain developed by Okamoto and Aoki (1963) Unop rated normotensive male Wistar rats served as controls in the experiments All rats received standard rat pell ts (Hankkija Oy Helsinki) and tap water *ad libitum* The systolic blood pressure of un anesthetized rats was measured with the tail cuff method of Friedman and Freed (1949) The details of the mod f cation us d ha e been described previously by us (Karppanen *et al* 1973 a)

Five weeks after the operative procedures the rats were sacrificed by decapitation Blood was collected for the determ nation of sodium and potassium by flame photometry and for the assay of plasma renin acti ity with the b ological method of Boucher *et al* (1964) The adrenal glands were quickly removed into ice cold Krebs Ringer b carbonate buffer prepared free from fat and quartered with a razor blade The details of the incubation of adrenal quarters as well as the measurement of aldosterone with a ga chro- m tograph c method ha e been described pre ously by Erbler (197) The following drugs were used ACTH (Acethropan® Farbwerke Hoechst AG Frankfurt Hoechst) N⁶ 2 O-dibutyryl cyclic adenosine 3 5 monophosphate (DBA, Boehringer Ma nheim GmbH Mannheim Waldhof) 5 hydroxytryptamine creat nine sulphate (Fluka AG Buchs)

Student s t test was used in the statistical analysis of the results

Results

Blood pressure (Fig 1)

5 weeks after the operation when the adrenal glands were taken for in vitro experiments the blood pressure of the pinealectomized rats was on the average 16 mmHg higher than the blood pressure of the control rats ($p < 0.01$) The blood pressure of those rats which had undergone the renal operation was 35 mmHg higher ($p < 0.001$) and that of the spontaneo usly hypertensive rats 57 mmHg higher ($p < 0.001$) than in the unoperated control rats

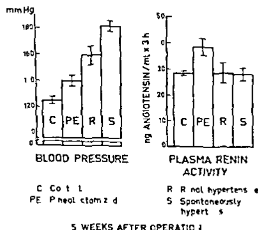


Fig 1 Blood pressure and pl sma renin ac ti ity n rats with p nealectomy induced re nal or spontaneous hypert nsion The alues were measured 5 weeks after p nealectomy or the removal of the right kid ey and tight n ng a f gure of e ght l gature around the left kidney

TABLE I Effect of ACTH DBA and 5HT on the release of aldosterone from rat adrenal quarters in vitro

Stimulant	Control	PE	RH	SH
None	0 47±0 02	0 38±0 06	0 51±0 09	0 46±0 07
ACTH 5×10⁻⁷ M	1 21±0 07	1 14±0 15	1 45±0 18	1 04±0 08[a]
DBA 5 10⁻⁴ M	1 [illegible]±0 15	0 84±0 05[c]	1 48±0 06	0 76±0 16[b]
5HT 1×10⁻⁶ M	0 89±0 05	0 85±0 04	0 85±0 04	1 03±0 14

The values indicate the release of aldosterone in μg per mg N during the 2 h incubation Means ± S E of 4 5 incubations are given
PE = pinealectomized RH = renal hypertensive SH = spontaneously hypertensive rats
[a] p < 0 05 vs renal hypertensive rats
[b] p < 0 01 vs renal hypertensive rats
[c] p < 0 001 vs renal hypertensive rats

Plasma renin activity (Fig 1)

5 weeks after the operation the plasma renin activity of the pinealectomized rats was increased by 35% (p < 0 01) In the rats with renal or spontaneous hypertension the plasma renin activity did not differ from control values

Production of aldosterone (Table I)

The basal release of aldosterone from the adrenal quarters was similar in all groups ACTH (5 10 M) DBA (5 10⁻⁴ M) as well as 5 HT (1 10⁻⁶ M) significantly increased the release of aldosterone (p < 0 05 to < 0 001) The response to ACTH and DBA was greater the adrenal glands of the rats with renal hypertension than in those of the spontaneously hypertensive or pinealectomized rats The response to 5HT was similar in all groups

Plasma electrolytes (Table II)

The plasma sodium concentration was similar in all groups In the rats with renal hypertension the plasma potassium was decreased (p < 0 001) as compared to the unoperated control rats In the pinealectomized and spontaneously hypertensive rats the plasma potassium concentration was unchanged

TABLE II Plasma sodium and potassium in rats with pinealectomy induced renal or spontaneous hypertension

Rat group	Plasma sodium mEq l (mean ± S E)	Plasma potassium mEq l (mean ± S E)	n
Control	137 ± 1	5 1 ± 0 1	14
Pinealectomized	139 ± [illegible]	5 [illegible] ± 0 1	10
Renal hypertensive	133 ± [illegible]	4 6 ± 0 1	10
Spontaneously hypertensive	139 ± 1	4 9 ± 0 1	8

The electrolytes were determined 5 weeks after pinealectomy or the removal of the right kidney and tightening a figure of eight ligature around the left kidney
p < 0 001 vs unoperated control rats

Discussion

Several investigators have suggested that the renin aldosterone system may become relatively unimportant in the maintenance of chronic renovascular hypertension in animals with one kidney removed (Vapaatalo *et al* 1970 Brunner *et al* 1971 Krieger *et al* 1971 Pals *et al* 1971 Brunner *et al* 1972) In agreement with thi in the present study the plasma renin activity of rats with renal hypertension was at the level of the controls 5 weeks after the constriction of one kidney and contralateral nephrectomy However in dogs previously subjected to unilateral nephrectomy both plasma renin activity and arterial blood pressure rise within a few minutes after renal artery constriction (Gutmann *et al* 1972) Miller *et al* (1972) demonstrated that the increased plasma renin activity is responsible for the initiation of the elevated blood pressure in such dogs Experiments with specific angiotensin antagonists indicate that the renin angiotensin mechanism accounts for the elevation of blood pressure in the acute phase of renal hypertension also in rats (Pals and Fulton 1973) In the present study the adrenal glands of the rats with chronic renal hypertension showed an increased synthesis of aldosterone in response to stimulation with ACTH or DBA This may further point to the importance of the renin aldosterone mechanism in the renal hypertension In agreement with our previous works (Karppanen *et al* 1970 Karppanen *et al* 1973 b Karppanen 1974) the plasma renin activity of the pinealectomized rats was increased In the present work the plasma renin activity of the spontaneously hypertensive rats was found to be at the level of the control rats Sen *et al* (1972) reported that in the spontaneously hypertensive rats the plasma renin activity is age-dependent In young spontaneously hypertensive rats the plasma renin activity is increased (DeJong *et al* 1972 Sen *et al* 1972) but returns later to normal levels in rats with a body weight of over 200 g (Sen *et al* 1972) which corresponds to the weight of the rats of the present study

The increased plasma renin activity does not seem to be essential for the hypertension in the pinealectomized rats The beta adrenoceptor blocking drugs propranolol (Karppanen 1974) and tolamolol (Karppanen *et al* 1973 b) decreased the plasma renin activity of the pinealectomized rats However only propranolol exerted an antihypertensive effect despite the fact that the plasma renin activity was depressed more by tolamolol The synthesis of aldosterone in response to ACTH or DBA was diminished in the adrenal glands of the spontaneously hypertensive and pinealectomized rats as compared to the adrenal glands of the rats with renal hypertension This may suggest a relative unimportance of the renin aldosterone system at least in the established spontaneous and pinealectomy induced hypertension as compared to the renal hypertension

The sympathetic renal nerves are involved in the control of the release of renin from the juxtaglomerular cells (Birbari 1971) Both in the spontaneously hypertensive (Haeusler *et al* 1972 van Zwi ten 1973) and pinealectomized rats (Karppanen *et al* 1973 a) an increased sympathetic tone of central origin has been implicated in the pathogenesis of the hypertension It has been suggested that the increased plasma renin activity of the pinealectomized rats may be due to the increased activity of the sympathetic renal nerves (Karppanen *et al* 1973 a) This might also be the case in the spontaneously hypertensive rats

We suggest that the renin aldosterone system may be of crucial importance in the renal

TABLE I Effect of ACTH DBA and 5HT on the release of aldosterone from rat adrenal quarters in vitro

Stimulant	Control	PE	RH	SH
None	0.47±0.02	0.38±0.06	0.51±0.09	0.46±0.07
ACTH 5×10^{-7} M	1.21±0.07	1.14±0.15	1.45±0.18	1.04±0.08[a]
DBA $5\ 10^{-4}$ M	1.22±0.15	0.84±0.05[c]	1.48±0.06	0.76±0.16[b]
5HT 1×10^{-4} M	0.89±0.05	0.85±0.04	0.85±0.04	1.03±0.14

The values indicate the release of aldosterone in μg per mg N during the ? h incubation Means ± S E of 4–5 incubations are given
PE = pinealectomized RH = renal hypertensive SH = spontaneously hypertensive rats
[a] $p < 0.05$ vs renal hypertensive rats
[b] $p < 0.01$ vs renal hypertensive rats
[c] $p < 0.001$ vs renal hypertensive rats

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TABLE II Plasma sodium and potassium in rats with pinealectomy induced renal or spontaneous hypertension

Rat group	Plasma sodium mEq l (mean ± S E)	Plasma potassium mEq l (mean ± S E.)	n
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Pinealectomized	139 ± [illegible]	5.[illegible] ± 0.1	10
Renal hypertensive	133 ± [illegible]	4.6 ± 0.1	10
Spontaneously hypertensive	139 ± 1	4.9 ± 0.1	8

The electrolytes were determined 5 weeks after pinealectomy or the removal of the right kidney and tightening a figure of eight ligature around the left kidney
p 0.001 vs unoperated control rats

Acta physiol scand 1975 94 189–197

From the Institute of Physiology and Institute of Psychology University of Helsinki Finland

Optimal Parameters for Eliciting Cardio-Acceleration by Electrical Stimulation of the Ventromedial Hypothalamus

By

G Johansson R Kalimo T Paakkönen and S Ruusunen

Received 19 December 1974

Abstract

Johansson G R Kalimo T Paakkönen and S Ruusunen *Optimal parameters for eliciting cardio-acceleration by electrical stimulation of the ventromedial hypothalamus* Acta physiol scand 1975 *94* 189–197

The ventromedial hypothalamus of the unrestrained cat was stimulated electrically through permanent electrodes The stimulation parameters amplitude duration and frequency of the square wave pulses were combined to produce a 20 increase of the resting heart rate value The total electric charge for each parametric combination was calculated The charge per stimulation (10 s) varied from about 1 to 100 micro-coulombs depending on the parametric combination used The response could therefore not be expressed as a function of the charge but depended on the individual parametric combinations The relation among the stimulation parameters and the total amount of electric charge of the pulses was estimated by the correlation technique The results suggested that about 60 of the variation in the electric charge resulting in the constant response was due to the variation of the pulse repetition frequency The optimal parameters for eliciting cardio acceleration in the cat by stimulation of the ventromedial hypothalamus seem to be pulse durations between 0 1 and 0 5 ms and pulse repetition frequencies below 100 P/s with pulse amplitude as the dependent variable

Key words Electrical stimulation parameters ventromedial hypothalamus cardio acceleration correlation analysis

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The parameters of electrical impulses applied to the hypothalamus have frequently been shown to influence the character and strength of the behavioral and physiological responses elicited (Keesey 1964 Cox and Valenstein 1969 Mogenson Gentil and Stevenson 1971 Wauquier Niemegeers and Geivers 1972 Johansson *et al* 1974) Some authors have suggested that parameters such as pulse repetition frequency (PRF) pulse duration (PD) and pulse amplitude (PA) can be subsumed under a common factor charge (Q) (Ward 1959 Keesey 1962 Huston Mills and Huston 1972) Keesey (1962) for example reported that the rate of hypothalamic self stimulation remained constant in spite of changes in the PRF and PA provided that the charge was constant In contrast the longer the PD the more charge was required to maintain a certain rate of response

Studies on parameters of hypothalamic stimulation have usually been carried out with

hypertension In the spontaneously hypertensive and pinealectomized rats the increased plasma renin activity may merely reflect an increased activity of the sympathetic nervous system which is suggested to be the main cause of hypertension in these rats

We are grat ful to H Erbler M D in the Department of Pharmacology Medical School of Hannover 3000-Hannover Kleefeld Federal Republic of Germany for the kind measurement of aldosterone
Supported by a grant from the Y Jahnsson Foundation Finland

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Histology At the conclusion of the experiments the animals were killed their brains fixed in 10 neutral formaldehyde and the nuclei stained with Nissl stain (Romeis 1948) The posit ons of the elec trode tips were then localized under a dissecting microscope In 10 of the cats the e ectrodes had reached the ventromedial nucleus of the hypothalamus In one the electrode had been situated in the dorsal nucleus of the hypothalamus and in one in the periventricular arcuate nucleus Further in one animal the site of the electrode t ps could not be traced with certainty and in two no histological examination was made

ECG record g and basic heart rate (BHR)

The electrodes (EEG electrodes of cup type) for the ECG recording were fixed to the chest of the cat with a rubber g rdle Electrode paste was used to increase the contact with the skin The apparatus for the recordings was a Mingograf 4 B (Elema Schonander Sweden)

The cat was put into the observation box for 15 min after which 10-s ECG samples were recorded at one min intervals The mean of 10 successive recordings in the reclining but alert animal with a varia tion of ± 10 at most was called the BHR

Con ta t heart r te respon e (CR)

After the BHR had been determined stimulations were applied at intervals of at least 5 min It was checked that the ECG was always within the limits of the BHR before another stimulation was applied Stimulation was only given when the animal was reclining but not asleep and never if it was moving The train durat on of the stimulation was 10 s The heart rate response elicited was cal ulated 5 s after the beginning of stimulation A 0 increase in the BHR was called the CR

Expe im ntal sit ti n 1

In these xperiments on 14 animals the CR was elicited by keeping the PD constant at 1 ms PRFs of 10 50 100 and 500 Hz were combined with d fferent PAs until the CR was obtained at each frequency The PA was changed in steps at 5 min intervals as described above

Expe imental situat on 2

PRFs of 10 50 100 and 500 Hz were combined stepwise with pulses of different durations The PA was kept constant at a value which had been determined for each cat on the basis of pilot tests It was the lowest PA which resulted in agonistic behavior (f Nakao 1958 and Roman uk 1965) when the PRF was kept at 50 Hz a d the PD at 1 ms This value varied in the 6 animals used in these experiments from 0 1 to 0 5 mA (mean 0.28 ± 0 14 mA)

Exp mental s t at on 3

The PRF in these experiments was kept constant at 50 Hz and pulses of 3 different durations 0 0 0 2 and 2.0 ms were combined with varying PAs to evoke the CR Eleven cats were stimulated in this e peri mental situation

Results

The electrical stimulation eliciting the CR was too weak to induce any visuable vegetative or somato-motor responses in the cat Stimulation intensities higher than those eliciting CR however resulted in agonistic behavior previously described by *e g* Nakao 1958 Romaniuk 1965 and Johansson *et al* (1974)

Experimental situation 1

The PA fell in a steep slope from PRF 10 through 50 to 100 Hz When the PRF was 500 Hz, higher PAs were again needed to obtain the CR in 5 of the 14 cats When analyzed by two-way analysis of variance (McNemar 1962 pp 284-296) the differences between the means of the PA in the categories of PRF used were statistically significant (p 0 001 Table 1 and Fig 1) The monotonically decreasing trend of the PA-PRF curve appeared

TABLE I Means and standard deviations of pulse amplitude and total electric charge by pulse frequency PD = 1 0 ms N = 14

Pulse repetition frequency (Hz)	10		50		100		500	
	M	S D	M	S D	M	S D	M	S D
Pulse amplitude (mA)	0 23	0 11	0 13	0 05	0 09	0 06	0 17	0 10
Electric charge (μC)	2 70	1 09	8 33	[illegible] 79	12 88	6 43	104 34	52 3

Analysis of variance (a) Amplitude F = 10 47 df = 3 39 p < 001 (b) Charge F = 50 [illegible] df = 3 39 p < 001

to be statistically significant (p < 0 05) in a directional nonparametric trend analysis (Ferguson 1965) with a monotonic relationship as a hypothesis

Q corresponding to the parametric combinations used showed a tendency to increase with PRF (p < 0 001 Fig 1) The differences in the means of the Q (Table I) were also found to be statistically significant (p < 0 001)

Experimental situation 2 The PD–PRF curves

In spite of considerable deviation at a PRF of 10 Hz, the decreasing tendency of the PD was obvious up to PRF 50 (Fig 2) In the PRF range 50–500 Hz differences in the PD were small However the differences among all the means were statistically significant (p < 0 01) The curve describing the interdependence of the PD and the PRF had a monotonically decreasing trend (p < 0 025) In the corresponding curve for Q the differences between the means were significant (p < 0 01) and so was the monotonically increasing trend (p < 0 05) The results of analysis of variance are shown in Table II

Experimental situation 3 The PA–PD curve

The PA decreased from PD 0 02 through 0 2 to 2 0 ms but the slope was steeper between PD 0 02 and 0 2 ms than from 0 2 ms on (Fig 3) The longer the PD the less the deviation of the PA The differences in the means of the PA were statistically significant (p < 0 001) and the probability that the above described trend of these means was due to chance is almost zero (p 0 0005) Concerning the Q–PD relationship the differences in the means of the Q were also significant (p 0 001 Table III) and so was the monotonically increasing trend of the Q with increasing PD (p < 0 001 Fig 3)

TABLE II Means and standard deviations of pulse duration and charge by pulse frequency PA constant N = 6

Pulse repetition frequency (Hz)	10		50		100		500	
	M	S D	M	S D	M	S D	M	S D
Pulse duration (ms)	0 88	0 38	0 8	0 11	0 9	0 17	0 9	0 [illegible]0
Electric charge (μC)	[illegible] 8	1 33	4 75	3 57	10 5	9 1	5 33	55 14

Analysis of variance (a) Duration F = 9 19 df = 3 15 p 01 (b) Charge F = 4 88 df = 3 15 p 05

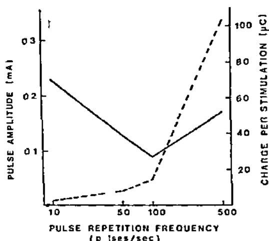


Fig. 1 Mean pulse amplitude (solid line surrounded by standard deviation) and electric charge per stimulation (dotted line) producing a 20 increase in the resting heart rate as functions of the pulse repetition frequency Pulse duration = 1 ms

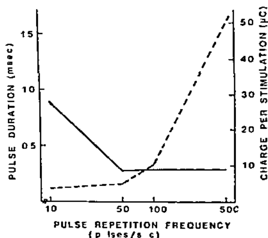


Fig. 2 Mean pulse duration (solid line surrounded by standard de iation) and electric charge per stimulat on (dotted line) producing a ∠0 increase in the resting heart rate as funct ons of the pulse repetition frequency Pulse ampl tude = constant

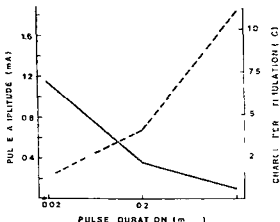


Fig 3 Mean pulse ampl t de (solid l ne surrounded by standard de iation) a d electric charge pe stimulat on (dotted l e) produ-ing a 0 crease in the rest ng heart rate as f ct ons of the pulse durat on Pulse repet tion freque cy = 50 Hz

TABLE III Means and standard deviations of pulse amplitude and charge by pulse duration. PRF = 50 Hz, N = 11

Pulse duration (ms)	0.02		0.2		2.0	
	M	SD	M	SD	M	SD
Pulse amplitude (mA)	1.15	0.40	0.36	0.21	0.09	0.08
Electric charge (μC)	1.37	0.43	3.98	2.22	12.15	9.72

Analysis of variance: (a) Amplitude $F = 83.46$, $df = 2, 20$, $p < 0.001$; (b) Charge $F = 13.47$, $df = 2, 20$, $p < 0.001$

Correlation analysis of the data

The relation between the Q necessary to elicit the CR and the PA, PD and PRF as well as the interrelations of these three parameters were analyzed by the correlation technique. The product moment correlations were calculated across the 3 experimental situations in which the CR was produced. Each stimulation resulting in the CR was considered as an observation unit. The number of units was 113. The dependence of Q on each separate parameter partialing out the effect of the other two parameters was analyzed with part and partial correlation techniques. Since the two techniques gave almost identical results the following considerations are based solely on the partial correlations.

Table IV lists the intercorrelations of PD, PRF, PA and Q. The correlations among the 3 first mentioned parameters were negative, 2 of them were low, but the correlation between PA and PD was quite high.

The correlation between Q and PRF was much stronger than the correlations of Q with the other parameters. Moreover, this was the only correlation that differed with statistical significance from zero ($p < 0.001$, two-directional test). Although not differing significantly from zero, the negative correlation between PA and Q is notable.

After adjustment for PRF, the product moment correlation 0.13 between PD and Q was 0.23. This value differs with statistical significance from zero ($p < 0.05$) on the basis of applications of the t test two directionally to the corresponding Z value of the partial correlation (Hays 1963, pp. 575–576). After the effect of PA was partialed out, the partial correlation was only 0.10. On the other hand, the corresponding partial correlation of the second order reached the value 0.31, which differs significantly from zero ($p < 0.01$, two-directional test). The correlations of PRF with PD and PA were quite low, for which reason the product moment correlation 0.76 between the PRF and Q changed little after

TABLE IV Intercorrelations of stimulation parameters. N = 113

		PD	PRF	PA	Q
Pulse duration (PD)	1				
Pulse repetition frequency (PRF)		0.03	—		
Pulse amplitude (PA)	3	0.56	0.14	—	
Electric charge (Q)	4	0.13	0.76	0.08	—

adjustments have been made for the other parameters It reached the value 0 78 in the partial correlation analysis of the second order

The initially low negative correlation between PA and Q reached a low positive value 0 04 after adjustment for PRF The change in the coefficient was smaller after adjust ment for PD the partial correlation being −0 01 Essentially different results were obtained when the effects of both PRF and PD were partialed out The partial correlation between PA and Q then reached the value 0 21 which differs statistically significantly from zero ($p < 0.05$ two directional test) However the coefficient remained lower than the corre sponding correlations between other parameters and Q

Discussion

The sensitivity of the heart rate to many overt stimuli as well as to movements of the body made strict control of the experimental conditions necessary It was observed in our experi ments that for example a rise from the lying position was enough to cause an increase in the heart rate of at least 20% The effect of mere changes in skeletal muscle tone associated with hypothalamically induced behavior (see *e g* Hunsperger 1969) was there fore minimized by making recordings only when the animal was reclining the heart rate then being in the range of the resting state On the other hand falling asleep caused a considerable decrement in the heart rate Therefore experiments were not made if the ani mal fell asleep It has been shown that heart rate alterations due to stimulation are different for gradually rising than for steeply rising bursts of pulses (Hyde 1966) This source of error was excluded by always applying the stimuli separately with stepwise changes

The considerable variation of the charge in the different parametric combinations capable of eliciting a constant heart rate reaction means that this stimulation response cannot be expressed as a function of the number of microcoulombs alone This is not in keeping with the findings of Keesey (1962) concerning self stimulated behavior Keesey concluded that this behavior could be expressed as a function of the charge It is notable that the charge in experimental situation 2 with a PRF of 500 Hz in combination with a constant PD of 1 ms required about twice as much charge as was needed in situation 1 with the same PRF in which PA was constant and PD the dependent variable In the latter situation the mean value of PD was 0 3 ms and the stimulation off period was consequently longer Thus most of the difference in microcoulombs required in these special cases probably can be explained by differences in stimulation thresholds due to the refractory periods of the neuronal elements (*cf* Abeles 1967) With PRFs of 100 Hz or less the lower efficacy of an increase of the PD than of PA probably is due chiefly to other factors such as differences in the size of the neuronal pool involved in eliciting the response (see *e g* Lullies 1961 Abeles 1967)

The correlation analysis showed that Q was essentially more sensitive to variation in PRF than in the other parameters Actually the results of the analysis (Table IV) suggest that about 60 of the variation in Q resulting in CR was due to variation in PRF An increase in PD or PA in the ranges used was much less effective in eliciting the CR When the results of the correlation analysis are interpreted however the way in which the

observation units were constructed in the present study must be taken into consideration If other values had been used for the parameters that were kept constant or at fixed values in each experimental situation the correlations probably would have been different

The charge required to evoke the constant heart rate response varied from 1 3 μC to 104 3 μC per stimulation depending on the parametric combination used (Table I II and III) The optimal combination when the aim was to obtain the lowest possible charge was PD = 0 02 ms PRF = 50 Hz and the mean of the dependent variable PA = 1 15 mA (Table III) The least effective combination was PD = 1 0 ms PRF = 500 Hz, and the mean of the dependent PA = 0 17 mA (Table I)

We suggest that the most physiological or optimal parameters for eliciting cardioacceleration in the cat by stimulation of the ventromedial hypothalamus are PDs between 0 1 and 0 5 ms and PRFs below 100 Hz with PA as the dependent variable Shorter PDs may require values of PA so great as to cause injury (Mickle 1961) The results do not permit generalizations about the parameters suitable for eliciting the numerous other autonomic and behavioral responses that can be elicited from the same brain area but may help in the choice of them

This research was supported by grants from the National Research Council for Medical Sciences Finland and from the Finnish Cultural Foundation

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Acta physiol scand 1975 94 198–221
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Facilitation from Contralateral Primary Afferents of Interneuronal Transmission in the Ia Inhibitory Pathway to Motoneurones

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Received 20 December 1974

Abstract

FEDINA L H HULTBORN and M ILLERT *Facilitation from contralateral primary afferents of interneuronal transmission in the Ia inhibitory pathway to motoneurones* Acta physiol scand 1975 94 198–221

The action of volleys in contralateral primary afferents on transmission in the Ia inhibitory pathways to motoneurones was investigated with intracellular recording from motoneurones. Ia IPSPs in flexor as well as most extensor motoneurones were regularly facilitated by volleys in contralateral high threshold muscle cutaneous and joint afferents in spinal cats under chloralose anaesthesia In decerebrate cats with a low pontine lesion transmission in Ia inhibitory pathways was not facilitated but rather depressed by volleys in these afferents The recurrent effects from motor axon collaterals were investigated on inhibitory transmission from different contralateral afferents to motoneurones. Previous investigations have shown that the interneurones mediating the reciprocal Ia inhibition receive recurrent inhibition via motor axon collaterals and Renshaw cells Now a strong positive correlation was revealed between recurrent depression of IPSPs evoked from different contralateral afferents and facilitation of Ia IPSPs by the same afferent volleys [illegible] that the recurrent depression of IPSPs from different contralateral primary [illegible] excitatory convergence onto the Ia inhibitory interneurones which [illegible] IPSP evoked in the motoneurone from these afferents

In a preceding paper Fedina and Hultborn (1972) analysed the effects on the interneuronal transmission in Ia inhibitory pathways to motoneurones by volleys in ipsilateral segmental afferents It was concluded that interneurones in the Ia inhibitory pathways to flexor as well as extensor motoneurones receive excitatory convergence from ipsilateral flexor reflex afferents (FRA) in spinal chloralose anaesthetized cats In decerebrate cats with a low pontine lesion (Holmqvist and Lundberg 1961) the transmission in that excitatory FRA pathway was blocked but an excitatory convergence from an apparently separate pathway from low threshold cutaneous afferents was still present

Antidromic impulses in motor axons were earlier shown (Hultborn Jankowska and Lindstrom 1971 a, b) to depress transmission in the Ia inhibitory pathway to motoneurones by

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[2] Supported by a grant from the Deutsche Forschungsgemeinschaft

a postsynaptic inhibition of the Ia inhibitory interneurones evoked via α motor axon collaterals and Renshaw cells Fedina and Hultborn (1972 cf also Hultborn *et al* 1971 a) investigated also the recurrent effects from motor axon collaterals on inhibitory transmission to motoneurones from other ipsilateral afferents A strong positive correlation was revealed between the recurrent depression of IPSPs evoked from different ipsilateral afferents and the ability of the same afferent volleys to facilitate Ia IPSPs This was taken to indicate that recurrent depression of IPSPs from different ipsilateral primary afferents reflect their excitatory convergence on the Ia inhibitory interneurones

The present investigation analyses in a similar way the facilitation of transmission in Ia inhibitory pathways to motoneurones by volleys in contralateral segmental afferents in different types of preparation The ability to evoke such a facilitation was then compared with the susceptibility to recurrent depression of the IPSPs evoked in motoneurones by the same contralateral volleys The systematic comparison has revealed a strong positive correlation between these events Our results thus support the suggestion by Hultborn *et al* (1971 a) that the recurrent inhibition of interneurones in afferent pathways to motoneurones is restricted to those mediating the reciprocal Ia inhibition to motoneurones

Methods

Most of the present material (derived from 6 cats totally) was collected from the same experiments presented in a preceding paper (Fedina and Hultborn 1972) dealing with the effect on transmission in the Ia inhibitory pathways by volleys in ipsilateral afferents The maintenance of the preparation and the experimental procedures followed that described in previous papers (Fedina and Hultborn 1972 Hultborn and Udo 197?) Ipsi and contralateral muscle and cutaneous nerves and a nerve branch from the knee joint were dissected and mounted on electrodes for stimulation These nerves are listed under Abbreviations

Four different preparations were used (see Fedina and Hultborn 197) I) unanaesthetized decerebrate decerebellate cats with a low pontine lesion—henceforth called unanaesthetized low pontine preparation (see Holmqvist and Lundberg 1961) II) same as in I) but with chloralose anesthesia (60 mg kg) III) unanaesthetized low spinal animals which were either decerebrated or anaemically decorticated IV) spinal animals with chloralose anaesthesia

The technique of stimulation of peripheral nerves and of intracellular recording from motoneurones has been described (Hultborn and Udo 197)

Averaging facilities In addition to the conventional recording of intracellular events the recorded potentials were fed to an averaging computer (CAT 1000) In order to minimize errors due to slow random variations in the potentials the unconditioned and conditioned responses were alternated and fed to different parts of the computer memory The averaged responses were displayed on an oscilloscope for photographing For a quantitative evaluation of the action of contralateral volleys on ipsilateral Ia IPSPs the use of a digitizing system (HP 9830) proved very helpful (*cf* Fig 5 and 6) Enlarged records of the intracellular potentials were traced with an analogue digital converter The digital values which were stored in a computer memory were used for various calculations Thus it was possible to subtract the different responses from one another and to take the integral over arbitrary parts of the recorded potentials These could be compared according to the conditioning testing situation

Abbreviations The following abbreviations are used anterior biceps and semimembranosus ABSm deep peroneus (without cutaneous and extensor digitorum brevis branches) DP gastrocnemius and soleus G S posterior biceps and semitendinosus PBSt extensor digitorum longus EDL flexor digitorum and hallucis longus FDL hamstring nerve (consisting of PBSt and ABSm) H posterior knee joint nerve J plantaris Pl quadriceps Q sartorius S suralis Sur tibialis (after giving branches to popliteus tibialis posterior and flexor digitorum and hallucis longus muscles) Tib flexor reflex afferents FRA ventral root VR postsynaptic potential PSP excitatory postsynaptic potential EPSP inhibitory postsynaptic potential IPSP recurrent inhibitory postsynaptic potential RIPSP dorsal root potential DRP primary afferent depolarization PAD ipsi and contralateral i and co

TABLE I Facilitation of Ia IPSPs by volleys in contralateral high threshold afferents The species of the motoneurones investigated are indicated to the left and the figures in parentheses indicate the number of cells tested The different preparations are given in the upper horizontal row column 1–4 Note that the preparations labelled decerebrate refer to decerebrate decerebellate cats with a low pontine lesion as described in Methods In each preparation the effect of stimulation of contralateral high threshold muscle afferents (coH) contralateral high threshold cutaneous afferents (coSur) and contralateral high threshold joint afferents (coj) are listed separately The figures presented for each nerve show the number of motoneurones in which a spatial facilitation was established of the total number of motoneurones tested in that combination (convergence/total) The sum of motoneurones recorded in the different preparations is greater than the number given in the parentheses on the left side since often different nerves were tested in one motoneurone In addition sometimes one motoneurone was tested in different preparations (cf Fig 6 and 8)

		1 Decerebrate unanesthetized			2 Decerebrate chloralose			3 Spinal unanesthetized			4 Spinal chloralose		
		coH	coSur	coj	coH	coSur	coj	coH	coSur	coj	coH	coSur	coj
Sart (7)	Convergence/total	—	—	—	—	—	—	—	—	—	6/6	4/4	—
PBSt (67)	Convergence total	0 10	1/10	—	0 9	0/3	—	2/9	1/7	?/	?7/30	4/?5	3/3
DP (11)	Convergence total	—	—	—	1/1	1/1	—	0/1	—	—	3/5	6 6	3 3
ABSm (19)	Converg nc /total	—	—	—	—	—	—	0 4	—	—	13/13	10/11	1/1
Q (38)	Convergence/total	0 8	0 1	—	0/2	0/1	—	0/5	1/3	—	10 15	9 9	—
G S (16)	Convergence/total	—	—	—	0/1	0/1	—	0 4	0/1	0/1	0/1?	1/9	0 4

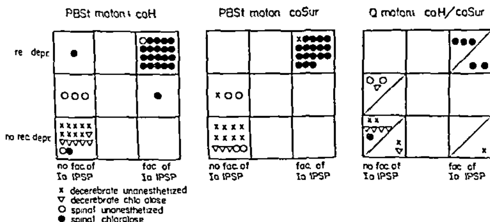
Results

The action of volleys in contralateral high threshold muscle afferents high threshold joint afferents and cutaneous afferents upon transmission in the ipsilateral Ia inhibitory pathways to different species of lumbar motoneurones are summarized in Table I In this table only the effects of volleys in coH coSur and coj are presented Other contralateral nerves (peroneal tibial and G S nerves) were also occasionally tested but no qualitative differences were found with the results in Table I The recurrent control by volleys in ipsilateral motor axon collaterals on transmission in the inhibitory pathways from the contralateral afferents to the motoneurones was also tested All ventral roots (L5 S1) besides those evoking a recurrent IPSP in the recorded motoneurone were then used for conditioning stimulation *It was always checked that the same conditioning volley depressed Ia IPSPs in the same cell This recurrent control was then correlated in individual motoneurones with the facilitation of* the ipsilateral Ia inhibition evoked by the same contralateral afferents Table II summarizes this correlation for the PBSt and Q motoneurones tested

1 *Spinal chloralose anesthetized preparation*

Flexors Fig. 1 illustrates two knee flexor PBSt motoneurones (A F G I) The test Ia IPSPs from Q (second trace in A D G) were conditioned by stimulation of nerves from the contralateral side When the conditioning contralateral volley itself evoked an IPSP (which was the usual case) the test Ia IPSP was ordinarily placed on its decay phase (as in Fig. 1 A D and G) to avoid the problems of a nonlinear summation of IPSPs and a conductance increase caused by the conditioning volley High threshold cutaneous afferents (coSur ..0 Th

TABLE II Comparison betwe n the susceptibility to recurrent depression of IPSPs from contralateral high threshold muscl afferents (coH) and contralateral high threshold cutaneous afferents (coSur) and the ability by volleys in the same afferents to facilitate transmission in the Ia inhibitory path way to PBSt and Q motor nuclei For each individual motoneurone the susceptibility to recur rent depression of IPSPs from the contralateral high threshold afferents has been plotted against the ability by volleys in the same afferents to facilitate a test Ia IPSP Different symbols indicate th type of preparation Note that the preparations labelled decerebrate refer to decrebrate dec rebellate cats with a low pontine l-sion Notice that the middle" groups (both at the abscissa and ordinate) means that it was not possible to decide if the effect was present or absent — no decision



high threshold muscle afferents (coH 25 T) and high threshold joint afferents (coj 50 T) effectively enhanced the test Ia IPSPs (third trace in the respective sets) This facilitation proves an excitatory action from these contralateral afferents on ip ilateral Ia inhibitory inter neurones which project to PBSt motoneurones The facilitatory action of volleys in contra lateral muscle and cutaneous afferents on transmission in the Ia inhibitory pathways some times became evident with stimulation strengths as low as 2 5–6 T (*i e* gr I muscle af ferents never contributed) However a strength of more than 10 T was often required and was always necessary with contralateral joint afferents In addition it proved sometimes useful to apply a short train (2–3 shocks) to demonstrate the facilitation Usually the facilita tion of Ia IPSPs increased with increasing stimulation strength up to about 50 T The contralateral muscle afferents (irrespective of muscle function) cutaneous and joint afferents facilitated the ipsilateral Ia inhibitory transmission without qualitative differences It is therefore reasonable to assume that the observed facilitation of the Ia IPSPs can be ascribed to an activation of a contralateral FRA pathway (R M Eccles and Lundberg 1959 a Holmqvist 1961)

A conditioning stimulation of L5 and L6 ventral roots depressed the facilitated Ia IPSP (Fig. 1 lowermost traces in A D G) In some cases it was nearly abolished by the ventral root stimulation (for example Fig 8 D) This demonstrates the convergence of excitation from contralateral FRA and inhibition from motor axon collaterals on the *same* Ia inhibi tory interneurones (see Hultborn and Udo 1972, pp 97–98 for a detailed description of this technique) It should be noticed that the FRA actions described here must be mediated by segmental pathways since the spinal cord was transected at low thoracic level

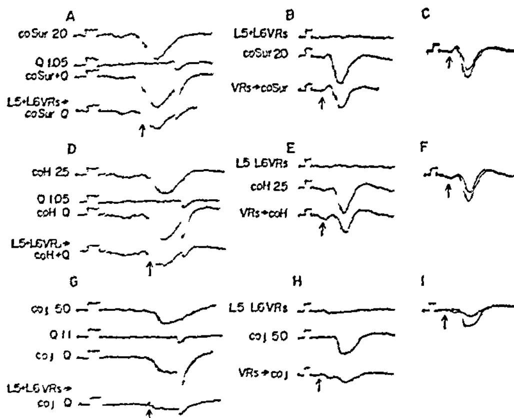


Fig. 1. Facil tation of Ia IPSPs in knee flexor motoneurones (PBSt) by volleys in contralateral h gh thresh ld aff rents and concomitant recurrent depression of the IPSPs evoked from the same contralateral h th e h d afferents. Chloralose anesthetized spinal cat. Two motoneurones (A F, G I). All traces are aver g d tracellular responses. In th s and following pictures the positivity is upwards for the intra ellular cord Cal bration pulses 1 mV 4 ms. The motoneurone of A F was depolarized by a current f 7(A. Th left column (A D G) shows the cond tion ng effect by volleys in coSur (A) coH (D) and coJ (G) th Ia IPSPs. The st mulation strength is ind cated in multiples of threshold for t e fastest af ferent fib es. Th recu re t effect f VR st mulation (ind cated by a rows) on the cond t oned Ia IPSPs is tested the l w rmost traces. In the m ddl column (B E H) the effect of VR stimulat on on the IPSPs evoked f m the respective contralateral ner es is illustrated. The cond tioned and unc nd t oned contralateral IPSPs a e supe imposed in the r ght column (C F I).

The records B E and H (Fig. 1) illustrate the action of a ventral root stimulation on the IPSPs which were evoked from the coFRA. The superposition of the conditioned and un conditioned traces in C F and I shows an effective depression of these IPSPs by a preced ing ventral root stimulation. Pro ided that the recurrently depressed parts of IPSPs in fact are mediated by Ia inhibitory interneurones this finding would indicate that at least to some extent the Ia inhibitory interneurones relay the IPSPs evoked in PBSt motoneurones by contralat ral FRA (see Discu on).

The facilitation of Ia IPSP by coFRA has been tested in different species of flexor moto neu ones. It can be seen fr m Table I that in the spinal preparation anaesthetized with chlo alose the facil tation was p esent in hip knee and ankle flexor motoneurones. Fig. 2

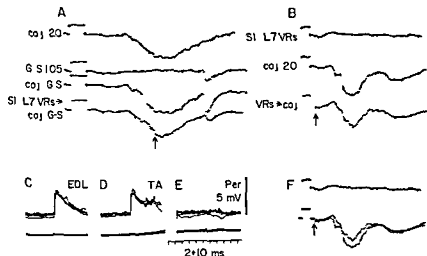


Fig 2 Facilitation of the Ia IPSP in a pretibial flexor motoneurone (DP) by volleys in the contralateral joint afferents and concomitant recurrent depression of the IPSP evoked from the same contralateral joint afferents Chloralose anaesthetized spinal cat. The motoneurone was depolarized by a current of 70 nA In C E showing the identificat on of the neurone the upper traces are intracellular potentials and the lower traces are recorded from the L7 S1 dorsal root entry zone In the surface lead in this and the following figures the negativity is upwards Time and voltage calibration for C–E are indicated in E The traces in A B and F are averag d intracellular responses Calibration pulses 1 mV 4 ms The time of stimulation of VRs is marked by arrows A shows the cond tioning effect of coj stimulation on the Ia IPSP In the lowermost trace L7 S1 VRs are st mulated additionally In B the recurrent effect of VR stimulation on the c j IPSP is shown The conditioned and uncond tioned IPSPs are superimposed in F

exemplifies the effects found in pretibial flexor motoneurones In a DP motoneurone (identification in C–E) the Ia IPSP from G–S (1 05 T) was enhanced by conditioning stimulation of coj (20 T A) and the IPSP by the coj volley was depressed by a condition ing ventral root volley (B F) just as illustrated for PBSt motoneurones in Fig 1 Facilita tion of Ia IPSPs was observed in 10 out of 11 investigated DP motoneurones and was also noticed in one Per motoneurone (not included in Table I) Two of the DP motoneurones were identified antidromically from the TA nerve (in an experiment with an intact L6 ventral root) However although a facilitation of the Ia IPSPs in DP motoneurones was found regularly in our limited material (Table I) it was obvious that this facilitation was not as pronounced as that found in knee flexor motoneurones

The records of Fig 3 illustrate the time course of the facilitation of a Ia IPSP evoked by a conditioning stimulation of the coSur nerve In a hip flexor Sart motoneurone stimula tion of the nerve from the ABSm muscle evoked a minute test IPSP (A faster sweep speed in the lower two traces) A conditioning volley in the coSur preceding the test stimuli with different time intervals facilitated the test IPSP (A–D) A conditioning ventral root stimula tion decreased the facilitated IPSP (E) As can be seen from the specimen records (B–D) the degree of facilitation was dependent on the interval between conditioning and test stimulation The full time course of the facilitation in this cell is shown in F The latency and time course of the facilitation of Ia IPSP from contralateral high threshold muscle

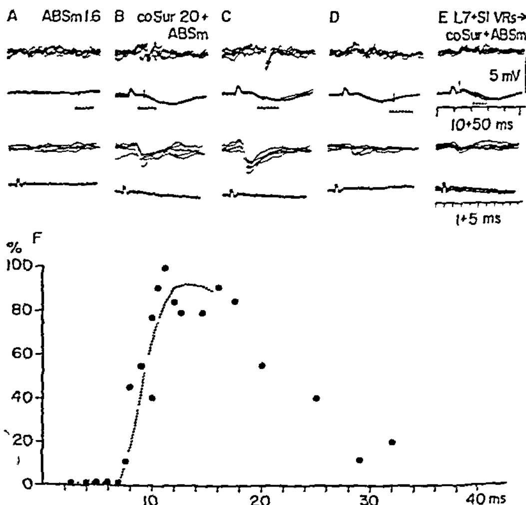


Fig. 3. Time course of the facilitatory action by contralateral high threshold cutaneous volleys on a Ia IPSP in a Sart motoneurone. Chloralose anaesthetized spinal cat. The motoneurone was depolarized by a current of 100 nA. In A–E the conditioned and unconditioned Ia IPSPs were recorded simultaneously at a slow and a fast sweep speed. The dashed lines below the upper slow records show the parts which are expanded in the lower records. In the respective pairs the upper traces are intracellular responses and the lower traces are recorded from the L6 dorsal root entry zone. Time calibration as indicated in E. The voltage calibration refers to the intracellular potentials. A–D give specimen records of the facilitatory time course. In E the facilitated IPSP was abolished following a conditioning volley in S1–L7 VRs. In F the time course of the facilitatory effect is plotted. The abscissa gives the interval between the arrival of the fastest conducting fibres (measured by the initial positivity in the surface lead) of the conditioning and testing volleys. Since the test stimulation elicited no Ia IPSP (A) the ordinate gives the amplitude of the facilitated Ia IPSPs in percent of the maximal Ia IPSP (11 msec conditioning testing interval) which was set to 100.

afferents is approximately the same as illustrated in Fig. 3 for cutaneous afferents. However the threshold for the "direct" postsynaptic potential by coFRA stimulation was often lower than the threshold to evoke a facilitation of Ia IPSPs. This made it difficult and often impossible to investigate the time course. In a forthcoming paper using direct recording of the

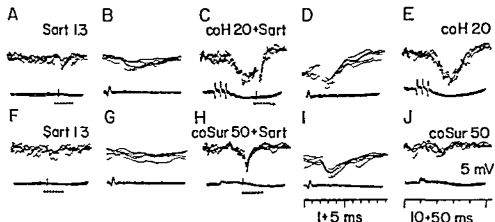


Fig 4 Facilitation of a Ia IPSP in a hip extensor motoneurone (ABSm) by volleys in contralateral high threshold muscle and cutaneous afferents Chloralose anaesthetized spinal cat. The motoneurone was depolarized by a current of 70 nA The upper traces are intracellular responses The lower traces are recorded from L7 dorsal root entry zone The dashed lines below the slow traces show the parts which were expanded in the neighbouring records Time calibration for the slow records in J for the fast records in I The voltage calibration refers to the intracellular potentials

interneurones mediating reciprocal Ia inhibition (Hultborn Illert and Santini to be published) the latency and time course of the coFRA actions will be further illustrated

Extensors In hip knee and ankle extensor motoneurones excitation of contralateral high threshold afferents elicited EPSPs predominantly However current passage through the microelectrode revealed that volleys in the coFRA usually evoked an IPSP in addition to the dominating EPSP Following depolarization of the motoneuronal membrane it was therefore often possible to investigate in extensor motoneurones the action of coFRA upon the Ia inhibitory transmission as well as the recurrent control of the IPSPs evoked from the same contralateral afferents

Fig 4 illustrates an hip extensor ABSm motoneurone in which the membrane had been depolarized Volleys in contralateral high threshold afferents of muscular (coH 20 T) and also to some extent of cutaneous (coSur 50 T) origin evoked IPSPs (E and J) When the Ia IPSP was preceded by the contralateral FRA volleys it was effectively facilitated (*cf* A–D F–I) The increase of the test Ia IPSP appears particularly clear in the fast traces of D and I They suggest a convergence of coFRA excitation on the Ia inhibitory interneurones projecting to hip extensor muscles

Fig 5 illustrates a representative knee extensor Q motoneurone In the depolarized cell stimulation of the coSur nerve elicited an IPSP (upper trace in A) The Ia IPSP evoked by PBSt (second trace) was conditioned by a volley in the coSur (third trace) The dashed line in A indicates the part of the traces which is reproduced in B using an expanded time scale Superposition of the traces (see further in legend) shows that the volley in the coSur facilitated the Ia IPSP The records in C and D show that the IPSP evoked by the volley which facilitated the test Ia IPSP was depressed by antidromic volleys in L7 and S1 ventral roots (*cf* the superimposed traces of the unconditioned and conditioned IPSP in D)

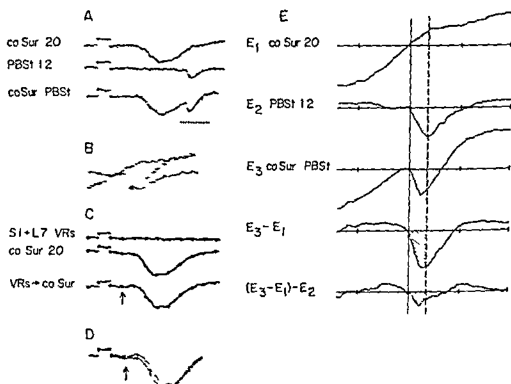


Fig 5 Comparison of susceptibility to recurrent depression of contralateral high threshold cutaneous IPSPs and ability by volleys in the same contralateral afferents to facilitate Ia IPSPs in a Q motoneurone Chloralose anaesthetized spinal cat. The motoneurone was depolarized by a current of 100 nA The traces A–D are averaged intracellular potentials Calibration pulses 2 mV 4 ms Th stimulation of VRs is i di ated by arrows A shows the cond tioning effect of a contralateral high threshold cutaneous volley (coSur) on the Ia IPSP The dashed line below the records indicates the part of the traces which is shown at an expanded time scal in B The a eraged intracellular responses after cond tioning and conditioning test stimulation are arranged in such a manner that the contralateral high threshold IPSPs are superimposed In addition to evaluate a possible facilitation of the Ia IPSP the unconditioned Ia IPSP is super imposed on the peak of the cond ti ned Ia IPSP E shows the quantit tive analysis of this fac l tation Further description in the text In C the effect of VR stimulation on th IPSP evoked from the contra lateral h gh threshold cutaneous afferents is illustrated The conditioned and uncond tioned IPSPs are sup rimposed in D

Stimulation of coFRA facilitated the transmission in the Ia inhibitory pathway to most Q motoneurones tested However Fig 5 illustrates one of the more evident examples of this excitatory convergence from coFRA on Ia inhibitory interneurones impinging on Q motoneurones—the action was usually weaker As can be seen in Fig 5 A the estimation of the amount of facilitation was therefore rendered more difficult since the test Ia IPSP had to be placed on the decay phase of the IPSP elicited by the contralateral nerve Therefore the potential evoked by the conditioning stimulation had to be subtracted from the response which wa due to the combined conditioning test stimulation

Fig 5 E shows the subtraction procedure as it has been performed in the Q cell of A D The curves of A were traced using an analogue-digital converter (cf Methods) E_1 and E show the unconditioned and conditioned Ia IPSP E shows the corresponding period of the

IPSP evoked by the conditioning coSur stimulation The reference point at the beginning of the unconditioned Ia IPSP is indicated in each trace by the crossing of the solid horizontal and vertical lines The fourth trace gives the calculated potential after subtracting E_1 from E_3 thus revealing the facilitated Ia IPSP in isolation The component which is added to the test Ia IPSP (E_2) by the conditioning stimulation is shown in the lowermost trace which is formed by a further subtraction of the unconditioned Ia IPSP (E_2) from the isolated conditioned Ia IPSP (E_3–E_1) To estimate the percent change of the Ia IPSP effectuated by the conditioning stimulation the potentials were integrated over a limited period from the beginning of the unconditioned Ia IPSP to its peak (indicated by the broken line) In the fourth trace the hatched area gives the integral of the conditioned Ia IPSP after subtracting E_1 from E This area was compared with the integral of the unconditioned IPSP which was set to 100% (dotted area in E_2) The hatched part in the lowermost trace marks the integral of the area which was added to the test Ia IPSP by the conditioning stimulation In this cell the conditioned Ia IPSP (E_3–E_1) was facilitated to 148% of the unconditioned test IPSP

All knee and ankle extensor motoneurones have been analysed according to this procedure Table I reveals a difference between these two groups of motoneurones Whereas in the spinal cat with chloralose anaesthesia the Ia IPSP to Q motoneurones was facilitated regularly by contralateral high threshold muscle and cutaneous afferents this facilitation was not found in the Ia inhibitory pathway to G–S motoneurones in the present conditions (*cf* Discussion)

2 *Spinal unanaesthetized preparation*

In the spinal unanaesthetized preparation volleys in contralateral high threshold afferents only rarely facilitated the Ia IPSPs (Table I) This finding applies as well to flexor as to extensor motoneurones Fig 6 illustrates a flexor motoneurone (PBSt) in a spinal preparation In the unanaesthetized state (A–B) the Ia IPSP from Q (1 1 T) was conditioned by stimulation of coH (20 T) The averaged records of A and the arithmetic procedure in B reveal that the conditioning stimulation had no effect on the test Ia IPSP However after intravenous injection of chloralose (60 mg/kg) a facilitation of the Ia IPSP became evident Now volleys in the coH greatly enhanced the test Ia IPSP (C and D) This proves the convergence of contralateral high threshold muscle afferents on ipsilateral Ia interneurones As an additional indication of this excitatory convergence the stimulation of the coH now decreased the time to peak of the test Ia IPSP (see D_1 and D –D)

3 *Decerebrate cats with a low pontine lesion (low pontine preparation)*

A suppression of coFRA actions is seen in the decerebrate state but after a medial low pontin lesion there is a release of inhibitory paths from coFRA to both flexor and extensor motoneurones while transmission in the excitatory paths is still suppressed (Holmqvist 1961 *cf* Holmqvist and Lundberg 1961 for the ipsilateral FRA) Originally it was therefore thought that this latter preparation henceforce referred to as the low pontine preparation would offer excellent possibilities for analysis of the inhibitory FRA pathways to motoneurones and its presumed mediation via the Ia inhibitory interneurones However

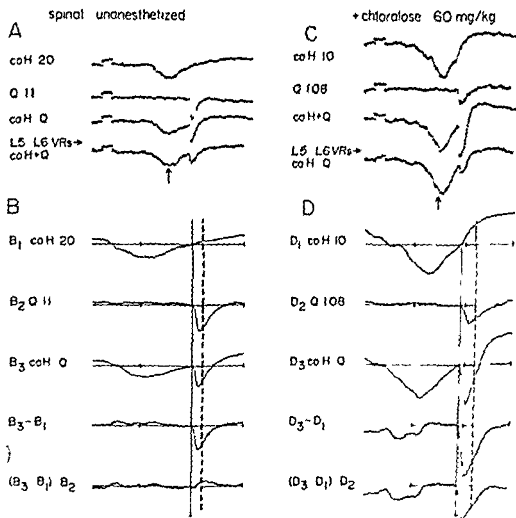


Fig. [illegible] Action of volleys in contralateral high threshold muscle afferents on Ia IPSPs in the spinalized state (A, B) and after administration of chloralose (C, D). The PBSt motoneurone was depolarized by [illegible] 50 nA. A and C are averaged intracellular responses. Calibration pulses 1 mV, 4 ms. Stimul[illegible] VRs is marked by arrows. In A and C the conditioning effect by volleys in coH on the Ia IPSP [illegible]. The lowermost traces illustrate the effect of an additional VR stimulation on the conditioned Ia IPSP. B and D give the quantitative analysis of the conditioning action of coH on the Ia IPSP. Further d[illegible] the text.

preceding investigation on ipsilateral FRA pathways (Fedina and Hultborn 1972) surprisingly revealed that although volleys in ipsilateral FRA evoke IPSPs in all motoneurones in such preparations there were never any sign of facilitation of Ia IPSPs.

Fig. 7A, C exemplifies the action of contralateral high threshold muscle afferents in an unanaesthetized low pontine preparation. In a PBSt motoneurone the Ia IPSP was elicited by stimulation of the Q nerve (Fig. 7A). Conditioning stimulation of coH (50 T) which evoked an IPSP in the motoneurone did not increase but rather decreased the a[illegible]

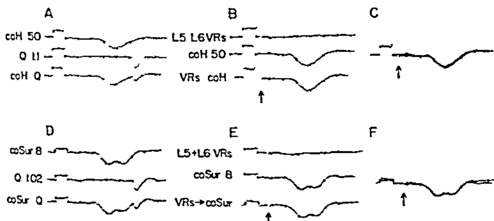


Fig. 7 Comparison of susceptibility to recurrent depression of contralateral high threshold muscle and contralateral high threshold cutaneous IPSPs and ability by volleys in the same contralateral afferents to facilitate Ia IPSPs in the decerebrate unanaesthetized state. Two PBSt motoneurones (A–C 60 nA depolarized D F 56 nA depolarized) were recorded in the same animal All traces are averaged intracellular responses Calibration pulses 1 mV for A–C . mV for D–F 4 ms The stimulation of VRs is indicated by arrows A and D show the conditioning effect of high threshold volleys in coH (A) and coSur (D) on the Ia IPSPs In B and E the effect of VR stimulation on the IPSPs evoked from the contralateral nerves is illustrated The conditioned and unconditioned contralateral IPSPs are superimposed in C and F

of the Ia IPSP In B and C (Fig. 7) the recurrent control of the IPSP evoked by the contralateral afferents was investigated Antidromic volleys in the L5–L6 ventral roots had no effect on this contralateral IPSP as can clearly be seen from the superimposed conditioned and unconditioned traces in C Similarly as in flexor motoneurones it was found that volleys in high threshold muscle afferents did not facilitate the Ia IPSP in extensor motoneurones (Table I) and that IPSPs evoked from the same afferents were unaffected by antidromic volleys (Table II)

Fedina and Hultborn (1972) reported that in the low pontine preparation ipsilateral cutaneous afferents of low threshold did facilitate Ia IPSPs thus in contrast with the high threshold muscle and joint afferents However as can be seen from Table I in the low pontine preparations the contralateral cutaneous afferents did not act independently from high threshold muscle afferents This is illustrated for a PBSt motoneurone in Fig 7 D Records in E–F (see also Table II) show that the coSur IPSP itself was unaffected by conditioning antidromic volleys in L5 and L6 ventral roots which effectively depressed the Ia IPSP in the same cell (not shown) The lack of recurrent depression of inhibition from contralateral skin afferents to PBSt and Q motor nuclei was also confirmed by the use of monosynaptic test reflexes (6 cats) This was in sharp contrast to the effective recurrent depression of ipsilateral cutaneous inhibition of monosynaptic test reflexes in the same preparations (cf Fig 10 in Fedina and Hultborn 1972)

In the unanaesthetized spinal state the facilitation of Ia IPSPs by contralateral FRA was rather infrequent but was observed regularly after administration of chloralose (see above) Thus the question arose whether the failure to facilitate Ia IPSP in the unanesthetized low pontine cat was due to the absence of the chloralose anaesthesia However a com

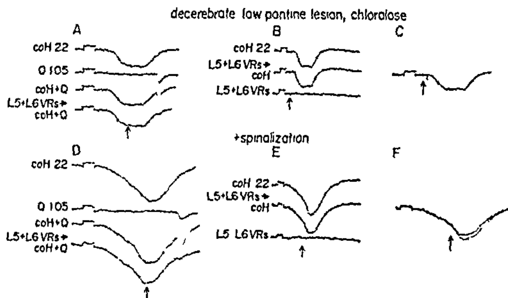


Fig. 8. Comparison of susceptibility to recurrent depression of contralateral high threshold muscle IPSPs and ability by volleys in the same contralateral afferents to facilitate Ia IPSPs in the decerebrate and the spinal state with chloralose anaesthesia. The PBSt motoneurone recorded in the decerebrate state with a low pontine lesion (A–C) and after subsequent spinalization (D–F) was depolarized by 70 nA. All traces are averaged intracellular responses. Calibration pulses 0.5 mV, 4 ms. The stimulation of VRs is indicated by arrows. A and D show the conditioning effect of high threshold volleys in coH on the Ia H SP. The recurrent effect of VR stimulation is shown in the lowermost traces. In B and E the effect of VR stimulation on the IPSPs evoked from the coH is illustrated. The conditioned and unconditioned contralateral IPSPs are superimposed in C and F.

parison between column 1 and column 2 in Table I reveals that in the low pontine preparation the additional presence of chloralose had no influence on the coFRA action on Ia IPSPs. Fig. 8 illustrates this point for a PBSt motoneurone which was first recorded in the chloralose anaesthetized low pontine state and then continuously during and after a complete spinalization. In the low pontine state (A–C) the Ia IPSP was not influenced by the conditioning stimulation of contralateral high threshold muscle afferents (A). B and C demonstrate that in addition the transmission in the inhibitory path from coH to the motoneurone was not controlled by antidromic volleys in motor axon collaterals. After spinalization (D–F), however, the Ia IPSP was effectively facilitated by the same conditioning volley in the coH (D). The additional stimulation of L5–L6 VRs nearly abolished the facilitated Ia IPSP thus demonstrating the convergence of coFRA and recurrent inhibition on the same Ia inhibitory interneurones. Furthermore, now the transmission in the inhibitory pathway from coH to motoneurones was depressed by volleys in the recurrent motor axon collaterals (E–F). The superposition of the unconditioned and conditioned traces in F shows a distinct depression of the conditioned IPSP. These results clearly demonstrate that in the low pontine preparation (unanaesthetized or with chloralose anesthesia), the transmission in the excitatory path from coFRA to Ia inhibitory interneurones is depressed.

In the decerebrate state with a low pontine lesion it was thus a consistent finding that volleys in the contralateral nerves did not facilitate the test Ia IPSP (Table I). On the con-

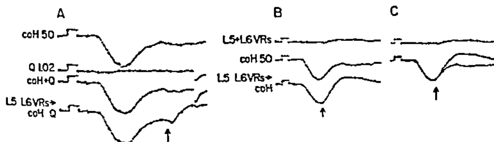


Fig 9 Late facilitation of a Ia IPSP by conditioning volleys in contralateral high threshold muscle af f rents in the d-cerebrate unanaesthetized state and concomitant recurrent depression of the late parts of the IPSP evoked by the sam contralateral muscle nerve The PBSt motoneurone was depolarized by 17 nA All traces are averaged intracellular responses Calibration pulses 1 mV 4 ms Stimulation of VRs marked by arrows In A the conditioning effect of volleys in coH on the Ia IPSP is shown The lowermost trace illustrates the effect of an additional stimulation of VRs In B the effect of VR stimulation on the IPSP evoked from the contralateral high threshold muscl afferents is illustrated The conditioned and un conditioned IPSPs are superimposed in C.

trary they often reduced the amplitude of the Ia IPSPs (Fig 7 A and D) By measuring the input resistance of the motoneurones (Eide 1968) it was often possible to exclude that a conductance change in the motoneuronal membrane was reponsible for the decreased ampli tude of th- IPSP Further it was always .scertained that the depression o the test Ia IPSP was not caused by recurrent inhibition secondary to a discharge of motoneurones follow ing the coFRA volley (no discharge was elicited in tne ventral roots) Thus the results in dicate that in the low pontine preparation volleys in contralateral high threshold muscle and cutaneous afferents may depress the transmission in the Ia inhibitory pathways to motoneurones The inhibition from coFRA of the interneurones mediating the reciprocal Ia inhibition will be dealt with in more detail in a forthcoming paper using direct recording from these interneurones (Hultborn Illert and Santini to be published)

In two exc ptional unanaesthetized low pontine preparations stimulation of coFRA el ited two phases of inh bition The early IPSP (with an approximate c ntral latency"—measured from the group I in com n volley—of about 5–6 msec) corresponds to the IPSPs illustrated in Fig 7 A–C D–F and Fig 8 A These "early" coFRA IPSPs were ne er depressed by condition ng olleys in the ventral roots and there was no facil tation of Ia transmission during this period (see abo e) Howe er dur ng the occas o al "lat " phase (central latency of about 30–35 msec) a fac litation of Ia inhibition was found (Fig 9 A) tog-ther w th a susc ptib l ty to recurrent d pression (F g 9 B C) This late response might well ha e been du to some sp no bulbo spinal reflexes (Shim mura 1963 Shimamura and L ngstone 1963) It d sappeared with sp nal transect on Thes late coFRA effects are not incl ded i Tables I and II

4 *Recurrent depression correlated with facilitation of Ia IPSP*

In spinal cats it was often observed that IPSPs evoked by volleys in coFRA which f.cilitated the Ia IPSPs themselves were depressed by antidromic volleys in motor axon collaterals (Fig 1 2 5 8) This was interpreted as a sign that the coFRA IPSPs at least partly wer mediated by the Ia inhibitory intern-urones If this holds true one should expect a syste matic correlation between the facilitation of Ia IPSPs by coFRA and depression of the coFRA IPSPs by antidromic volleys in the recurrent motor axon collaterals Table II il lustrates this correlation for the material of PBSt and Q motoneurones For individual motoneurones the recurrent depression (ordinate) of IPSP from contralateral high thresh

old muscle afferents (coH) and from contralateral high threshold cutaneous afferents (coSur) is plotted against the ability by volleys in the same afferents to facilitate Ia IPSPs (abscissa) Each symbol represents one motoneurone and the table gives only the presence or absence of a recurrent depression and of a facilitation of a test Ia IPSP In several cases it was impossible to decide under which group the result should be classified Consequently they were omitted from further comparisons They are however presented as a middle group in order to illustrate the frequency at which a decision in any direction was impossible In the PBSt motoneurones stimulation of the coH revealed a strongly positive correlation between the susceptibility to recurrent depression and ability to facilitate Ia IPSPs There was neither facilitation of Ia IPSPs nor any depression of the high threshold muscle IPSPs by ventral root volleys in decerebrate cats with a low pontine lesion (unanaesthetized or with chloralose anaesthesia) In the spinal chloralose anaesthetized preparations volleys in these afferents facilitated the Ia IPSPs The IPSPs evoked by the same afferents were depressed by antidromic volleys

Stimulation of the coSur nerve demonstrated the same strong positive correlation as found for the contralateral muscle afferents A facilitation of the Ia IPSPs and a recurrent depression of the high threshold cutaneous IPSPs was only observed in spinal cats anesthetized with chloralose A similar relation was also established for the Q motoneurones (right diagram) in which the effects from coH and coSur are put together

In Table II there are two exceptions to the general rule of the parallelism between susceptibility to recurrent depression and ability to facilitate Ia IPSPs In one PBSt motoneurone stimulation of the coH nerve did not increase the test Ia IPSP although the coH IPSP itself was depressed by a ventral root stimulation (spinal chloralose preparation) However a failure to demonstrate spatial facilitation does not necessarily exclude an excitatory convergence on the Ia inhibitory interneurones—the failure can be caused by a small subliminal fringe which would tend to keep the occlusion (also a consequence of convergence) as large as or even larger than the facilitation (see further Fedina and Hultborn 1972 p 73)

In the other case (in an unanaesthetized low pontine preparation) a coSur volley facilitated the test Ia IPSP in a Q motoneurone while the coSur IPSP itself was not susceptible to recurrent depression This might first of all indicate some failure of the decerebrate control of the excitatory FRA pathways to the Ia inhibitory interneurones It is then reasonable to assume that under these conditions the coFRA volley only gives a subthreshold excitation of the Ia inhibitory interneurones implying that the entire coFRA IPSP recorded is due to activation of interneurones other than those mediating the reciprocal Ia inhibition (cf p 73 in Fedina and Hultborn 1972)

Considering the experimental difficulties the obtained correlation between susceptibility to recurrent depression of coFRA IPSPs and the ability by volleys in the same afferents to facilitate test Ia IPSPs must be regarded as remarkably good

Discussion

1 *Effects from contralateral FRA on interneurones mediating the reciprocal Ia inhibition*

Bruggencate and Lundberg (1974) and the present investigation show that a facilitation of transmission in Ia inhibitory pathways to motoneurones can be evoked by volleys in con

tralateral high threshold muscle afferents (from flexors as well as extensors) high threshold cutaneous afferents and high threshold joint afferents No qualitative differences could be discerned between the effects on Ia inhibitory transmission from these various afferents Low threshold afferents on the contrary were always ineffective This pattern of convergence has led us to conclude that the facilitation of Ia inhibitory transmission is exerted by a contralateral FRA pathway (*cf* Holmqvist 1961)

Previously Bruggencate *et al* (1969) and Bruggencate and Lundberg (1974) described this facilitation of Ia inhibitory transmission mainly for knee flexor motoneurones However in the present investigation (see also Safyants *et al* 1973) the facilitation of transmission was in addition established in motoneurones supplying some hip and ankle flexors Furthermore a similar facilitation was revealed in hip (ABSm) and knee extensor motoneurones although normally less spectacular than in the case of flexor motoneurones It was hard to find any corresponding facilitation of Ia IPSP from coFRA in ankle extensor motoneurones but its presence has recently been reported by Safyants *et al* (1973) using slightly different preparations Thus it can be concluded that volleys in contralateral FRA are able to facilitate transmission of Ia inhibition to flexor as well as extensor motoneurones This conclusion is also supported by direct recording from interneurones supposed to mediate the reciprocal Ia inhibition (Hultborn Illert and Santini to be published)

Although Ia inhibitory interneurones inhibiting either flexor or extensor motoneurones can thus be excited by volleys in coFRA the transmission in the responsible coFRA pathway seems to be very dependent on the type of preparation Facilitation of Ia IPSPs was regularly found in the spinal preparation after chloralose administration (with the exception of G–S motoneurones) but was rather uncommon in the unanaesthetized spinal state The less frequent facilitation in the unanesthetized state may depend on a weaker FRA excitation of the interposed interneurones in that preparation or reflect that the excitation is obscured by concomitant inhibitory action Such a concurrent depression cannot however be caused by an interference at primary afferent level since no PAD in Ia afferents is produced by coFRA volleys in spinal cats (Devanandan *et al* 1965)

In decerebrate cats with a low pontine lesion coFRA volleys evoke inhibition in flexor and extensor motoneurones (Holmqvist 1961) but coFRA volleys did not facilitate Ia inhibitory transmission in this preparation (unanesthetized or under chloralose anaesthesia, Fig 7 and 8) On the contrary the coFRA volleys sometimes evoked a depression of Ia IPSPs (Fig 7) which was caused neither by conductance or potential changes in the motoneurone recorded nor by any recurrent inhibition of the Ia inhibitory interneurones secondary to firing of motoneurones *The reduction of the Ia IPSPs cannot be explained by* presynaptic inhibition of transmission from Ia afferents since FRA volleys in decerebrate cats (also after low pontine lesions) do not evoke DRPs (Carpenter *et al* 1963) Accordingly our findings suggest that the Ia inhibitory interneurones—like the motoneurones (Holmqvist 1961)—receive FRA inhibition in the decerebrate preparations with a low pontine lesion

From this account it appears that the coFRA pathway which excites Ia inhibitory interneurones seems to be regulated in parallel with the corresponding iFRA pathway (Fedina and Hultborn 1972) Both are thus operating in the chloralose anaesthetized spinal state

and seem to be obliterated in the low pontine state (with or without chloralose) In the latter preparation activation of coFRA as well as iFRA instead may depress Ia inhibitory transmission presumably by inhibition of the Ia inhibitory interneurones

Fedina and Hultborn (1972) found that volleys in ipsilateral low threshold cutaneous fibres did facilitate Ia IPSPs also in the decerebrate low pontine state when there was no corresponding facilitation from high threshold muscle and joint afferents This was taken to indicate the existence of a separate reflex pathway from low threshold cutaneous afferents to motoneurones In the present study we have been unable to find any evidence for a similar separate crossed cutaneous pathway since all cutaneous effects (which could be evoked only by volleys in high threshold afferents) have conformed with the common coFRA pattern

2 *Are inhibitory effects from coFRA mediated by Ia inhibitory interneurones?*

The large facilitation of Ia IPSPs sometimes evoked by conditioning volleys in the FRA has raised the question to what extent inhibitory coFRA actions to motoneurones are conveyed by interneurones in the Ia inhibitory pathways To investigate this problem we have conditioned coFRA IPSPs by antidromic volleys in motor axons known to obliterate transmission via the Ia inhibitory interneurones to the concerned motoneurones The validity of using recurrent depression of IPSPs as a criteria for their transmission via Ia inhibitory interneurones has been extensively discussed for IPSPs from ipsilateral afferents (Fedina and Hultborn 1972) and more generally for segmental and descending IPSPs (Hultborn 1972)

The present finding of a full convergence of Ia excitation coFRA excitation and recurrent inhibition leads to the conclusion that if coFRA volleys alone are able to fire these interneurones (as is shown by direct interneuronal recording Hultborn Illert and Santini to be published) the resulting IPSPs in the target motoneurones would be susceptive to recurrent depression Assuming that all Ia inhibitory interneurones receive recurrent inhibition (see Hultborn *et al* 1971 b) then the lack of recurrent depression would exclude that the IPSP was transmitted via the Ia inhibitory interneurones Conversely a conclusion that the recurrently depressed coFRA IPSPs are mediated by the Ia inhibitory interneurones depends on the assumption that interneurones in other inhibitory pathways to the motoneurones are not susceptive to recurrent inhibition The evidence obtained supports this assumption since measurements from many motoneurones revealed a strong positive correlation between the occurrence of recurrent depression of coFRA IPSPs and facilitation of Ia IPSP (Table II) From these results it is concluded that recurrent inhibition of interneurones mediating crossed inhibition is restricted to the Ia inhibitory interneurones

Since it is known that Renshaw cells themselves receive recurrent inhibition (Ryall 1970 Ryall Piercy and Polosa 1971) it [illegible] necessa[illegible] d[illegible] the possibility that coFRA IPSPs which are susceptible to recurrent depression in fact may have been mediated via these interneurones A transmission via Renshaw cells can occur either by th[illegible] [illegible] excitation or secondly to firing of motoneurones In order to exclude the latter possibility it [illegible] important to control that the afferent volleys used to evoke the test IPSP in the motoneurones do not el[illegible] discharge in ventral roots The possibility that volleys in coFRA may fire Renshaw cells orthodromically is very small since activation of coFRA instead exerts a powerful inhibition of Renshaw cells (Wilson Talbot and Kato 1964 Hultborn and Illert unpublished) If a small

part of the IPSPs nevertheless was to b conveyed by Renshaw c lls it is unl kely that even that part would be effectively depressed by conditioning ventral root volleys since the inhibitory interaction between Renshaw cells seems to be weak (Hultborn *et al* 1971 c see their Fig 6)

We thus conclude that recurrent depression of IPSPs from contralateral afferents in motoneurones seems to indicate that—and also roughly to which extent—they are medi ated by the Ia inhibitory interneurones

The extent to which the FRA inhibition is conveyed by the Ia inhibitory interneurones gauged by its sensitivity to recurrent depression is summarized in Table III This proce dure of course assumes that transmission of recurrent inhibition to the Ia inhibitory inter neurones is effective in all types of preparation used As stated in Results it was therefore always controlled that recurrent conditioning volleys effectively depressed Ia IPSPs in the motoneurones investigated

Although in the spinal state the excitation of extensors and inhibition of flexors from the coFRA is often the dominant action (cf the pattern in the classic crossed extensor reflex Sherrington 1910) the existence of alternative excitatory and inhibitory pathways from coFRA to both flexor and extensor motoneurones has been demonstrated in this prepara tion nevertheless (Holmqvist 1961) and intracellular recording from motoneurones usually reveals a mixture of EPSPs and IPSPs (Bruggencate and Lundberg 1974 and present in vestigation) The regular and sometimes prominent recurrent depression of coFRA IPSPs in flexor motoneurones in the spinal chloralose state indicates that an appreciable part of them are conveyed by Ia inhibitory interneurones (*cf* Fig 1 2 and 8 D–F) However the coFRA IPSPs were never completely obliterated after conditioning ventral root volleys (sometimes only a marginal depression was seen) This led us to conclude that coFRA IPSPs in flexor motoneurones in the spinal chloralose anaesthetized state besides their me diation by Ia inhibitory interneurones in addition are conveyed by private FRA inter neurones (i e by interneurones which are not shared with Ia afferents) Using these criteria in extensor motoneurones it seems that only a small fraction of the coFRA inhibition is conveyed by the Ia inhibitory interneurones (*cf* Fig 5) as is also indicated by the fact that the amplitude of coFRA IPSPs may exceed many times the amplitude of the maximal Ia IPSP

In the spinal unanaesthetized preparation (not included in Table III) that part of the coFRA inhibition which is mediated by the Ia inhibitory interneurones seems to be smaller in flexor as well as in extensor motoneurones In this state it is however often difficult to judge quantitatively the susceptibility to recurrent depression since the conditioning anti dromic volleys themselves frequently evoke a recurrent facilitatory potential and a conduct ance decrease of the motoneuronal membrane (Wilson and Burgess 1962 Hultborn *et al* 1971 c)

In the low pontine state (with or without chloralose) coFRA give rise to inhibition in ex tensor as well as flexor motoneurones Since these IPSPs are never susceptive to recurrent depression they should be exclusively mediated by private FRA interneurones (i e which are not shared with Ia afferents *cf* Fig 7 and 8 A–C) This was to be expected already from the finding that volleys in coFRA in that type of preparation never facilitate but rather depress Ia inhibitory transmission

TABLE III The extent to which ipsilateral and contralateral FRA IPSPs in motoneurones are mediated by Ia inhibitory interneurones under different circumstances The extent to which the FRA IPSPs are transmitt d via Ia inhibitory interneurones is based on their susceptiveness to recurrent depression (ipsilat ral FRA IPSPs Fedina and Hultborn 1972 contralateral FRA IPSPs present investigation) See further in the text

Preparation		Ipsilateral FRA			Contralateral FRA		
		Main effect	Inhibition		Main effect	Inhibition	
			Ia inhib int	Private int		Ia inhib int	Private" int
Low pontine (with or without chloralose)	Extensors	Inhib	0	+ +	Inhib	0	
	Flexors	Inhib	0	+ +	Inhib	0	+
Spinal, chloralose	Extensors	Inhib	(+)	+ +	Excit	(+)	
	Flexors	Excit	+	+	Inhib	+	+

Safyants et al (1973) recently reached the conclusion that Ia inhibitory interneurones as a rule do not take an active part in inducing contralateral FRA IPSPs The preparations in their investigation were not identical to ours but probably rather comparable to our chloralose anaesthetized spinal state since facilitation of Ia inhibitory transmission was one of their most regular findings Although the differences in preparation preclude a detailed comparison their conclusion seems to rest on a too indirect experimental approach It shall be emphasized that our conclusions are not only based on the susceptibility to recurrent depression of coFRA IPSPs but are strongly supported by direct recording from interneurones supposed to mediate Ia inhibition to flexor and extensor motoneurones (Hultborn Illert and Santini to be published)

Table III also summarizes the extent to which ipsilateral FRA IPSPs are thought to be mediated by the Ia inhibitory interneurones as judged by their susceptibility to recurrent depression (Fedina and Hultborn 1972)

3 The pattern of direct FRA effects on motoneurones in relation to the pattern of FRA facilitation of Ia inhibitory transmission

It has been postulated (Hongo *et al* 1969 Lundberg 1970) that neuronal pathways which evoke α linked movements can achieve a coupling between excitation of agonists and inhibition of antagonists by exerting excitatory action not only in α and motoneurones to *the agonists but also in the Ia inhibitory interneurones impinging on the motoneurones of* the antagonists (see Fig 10 A) Notice that the similar convergence of direct and indirect (from the Ia afferents via the γ loop) effects governs the excitation of agonist α motoneurones and of the interneurones inhibiting the antagonist motoneurones hence the term α- linkage in the reciprocal inhibition (Hongo *et al* 1969) The servo-assistance given by the γ-loop (*cf* Matthews 1972 pp 546–611) will thus support not only the contraction of agonists but to the same degree the relaxation of antagonists

Activation of ipsilateral as well as contralateral FRA pathways seems often to evoke parallel effects on α and motoneurones (*cf* Grillner 1969) Since volleys in ipsilateral and *contralateral FRA may also evoke excitation of the Ia inhibitory interneurones* (Fedina and Hultborn 1972 Safyants *et al* 1973 Bruggencate and Lundberg 1974 and present study) it seems as if the FRA pathways may represent such a neuronal system with full α linkage

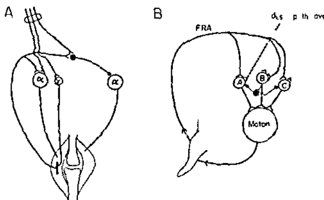


Fig 10 A Schematic representation of a neural pathway with parallel excitatory actions onto α and γ motoneurones to the agonists and Ia inhibitory interneurones impinging on the motoneurones of antagonists B Diagram showing alternative reflex pathways from the FRA (represented by the neurones A B and C) with different descending excitatory connexions (dashed lines) The inhibitory interactive connexions between the alternative FRA pathways are illustrated by the inhibition of pathways A and C from pathway B Several of the alternative FRA pathways may be organized to exert a full α–γ-linkage as illustrated in the diagram in A (The diagram in B is modified after Fig 4 in Lundberg 1973) See further in the text

giving a servo assisted control of both excitation and reciprocal inhibition Obviously it is only the reciprocal inhibition mediated by Ia interneurones which is of immediate relevance in the discussion of servo-assisted movements (*cf* Fig 10 A the private FRA inhibition will be considered later in this section) The dominating excitatory FRA action in the spinal chloralose preparation (see Table III) is exerted onto ipsilateral flexors and contralateral extensors and one would therefore rather expect a reciprocal inhibition (via Ia inhibitory interneurones *cf* Fig 10 A) mainly in ipsilateral extensor and contralateral flexor motoneurones It is *somewhat confusing in relation to this prediction that the most pronounced* inhibition via the Ia inhibitory interneurones is seen in flexor motoneurones with either ipsi or contralateral FRA activation (Table III)

The disquieting mismatch between expected and obtained pattern of FRA facilitation of Ia inhibitory transmission may well refer back to more basic questions concerning the organization of the FRA reflex pathways themselves It therefore seems important to review briefly some of the current ideas about the functional organization of these pathways (Lundberg 1973 Lundberg The FRA concept to be published see also in Bruggencate and Lundberg 1974) *The main theme includes the following points (cf Fig 10 B) (1) The* flexor reflex afferents (FRA) from muscles joints and skin (all with large receptive fields) seem to converge onto common interneurones The activation of the latter from the FRA may thus be essentially lacking space and modality specificity (2) Some of the afferents belonging to FRA are excited during normal limb movements and so do not seem related to any nociceptive reflex function (3) The FRA have several alternative reflex pathways to motoneurones and there is evidence for a mutual inhibitory action between (at least some of) them (4) The interneurones in (at least some of) the various alternative pathways can be activated from descending tracts (5) On the basis of these partly proved assumptions Lundberg (1973) proposed that selective activation (e g by descending command signals)

preparation It seems reasonable to assume that several discrete short latency FRA pathways are organized as to excite α and γ motoneurones to one group of muscles and the Ia inhibitory interneurones impinging on their antagonists as drawn in Fig 10 A In view of the complexities their final confirmation must await further experimentation

After these attempts to reconcile the observations on short latency FRA actions in the spinal cat with the scheme of a full α–γ linkage covering the reciprocal inhibition (Fig 10 A) it deserves to be stressed that many pathways may be organized along completely different principles There is for example now strong evidence for reciprocal inhibition which is *not* mediated via the Ia inhibitory interneurones (see *e g* Bruggencate and Lundberg 1974) If the assumption is accepted that inhibition mediated by the Ia inhibitory interneurones subserves α–γ linked movements it might be suggested that reciprocal inhibition mediated via private interneurones represents the reciprocal inhibition in α movements (Lundberg 1970 Hultborn 1972) However since the α motoneurones obviously are fired without the support of the γ loop in α movements nothing would refute that the same is true for the Ia inhibitory interneurones It is therefore not possible to exclude *a priori* that the Ia inhibitory interneurones may transmit reciprocal inhibition in α movements as well as in α–γ linked movements Be as it may reciprocal inhibition via private interneurones would be characterized by its dissociation from the servo-loop of the agonists (in α movements of course the servo-loop would not be operating at all) and by its independence of any recurrent control exerted by the active agonist motoneurones

Furthermore it shall not be taken for granted that all FRA actions are organized in a simple reciprocal manner there might well be pathways which increase or decrease excitability of motoneurones to extensors and flexors to different joints in much more complex patterns or perhaps even indiscriminately It is for example unknown to what extent the possibly non reciprocal low pontine FRA inhibition (see further below) is still present in the spinal state That pathway might be partly responsible for the FRA inhibition in motoneurones which is not mediated via the Ia inhibitory interneurones (present bilaterally in all species of motoneurones but particularly conspicuous in extensor motoneurones see Table III)

In contrast to the concurrent activation of several short latency FRA pathways discussed above it seems possible to obtain a *selective* transmission through a different set of FRA reflex pathways after i v injection of l DOPA in acute spinal cats (Andén *et al* 1966 Jankowska *et al* 1967) In this state a FRA volley evokes a long latency long lasting excitation of ipsilateral flexor and contralateral extensor α motoneurones (Andén *et al* 1966) and corresponding γ motoneurones (Grillner Hongo and Lundberg 1967 Bergmans and Grillner 1969 Grillner 1969) It was recently revealed (Fu Jankowska and Lundberg 1975) that the concomitant reciprocal inhibition in ipsilateral extensor and contralateral flexor motoneurones is induced mainly if not exclusively by Ia inhibitory interneurones The organization of this late FRA pathway seems thus to be a perfect example of the scheme in Fig 10 A

In decerebrate cats with a low pontine lesion (Holmqvist and Lundberg 1961 Holmqvist 1961) there seems to be a *selective* transmission through other inhibitory FRA pathways with an organization different from those discussed above The tonic descending inhibitory control of transmission in all FRA pathways seen in the intercollicularly decerebrate cat (R M Eccles and Lundberg 1959 b Engberg *et al* 1968 a b) is selectively released by a low

pontine lesion (Holmqvist and Lundberg 1961 Holmqvist 1961) so that stimulation of FRA then gives rise to a bilateral inhibition in flexor and extensor motoneurones Since there is no FRA excitation in the low pontine preparation the FRA inhibition in this state covering all species of motoneurones can hardly be regarded as reciprocal As would be expected from that point of view this FRA inhibition is never mediated by the Ia inhibitory interneurones (Table III)

It has to be admitted also that if the "low pontine" FRA inhibition is not "reciprocal" in this state it may nevertheless represent the inhibition which is reciprocal to the FRA excitation seen under other circumstances (e g in the acute spinal state in which it certainly a part of the FRA inhibition is mediated via private interneurones) That idea would assume an entirely differential control of an excitatory pathway and its inhibitory counterpart It seems more plausible to think of reciprocal inhibition as a "collateral" effect from their excitatory pathway at a rather late stage (for example from the last order interneurone in the excitatory pathway) This would of course not exclude a partial separate control of the reciprocal inhibition since there must be at least one interneurone interposed (the ones inhibiting the antagonist motoneurone) However with such a scheme it would be rather hard to imagine a full transmission through the reciprocally inhibitory paths while their excitatory counterparts are completely obliterated

Excellent technical assistance was given by Mrs Rauni Larsson This work was supported by the Swedish Medical Research Council (Projects No 94 and 4 48)

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Acta physiol scand 1975 94 222–228

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Prostaglandin Endoperoxides IX Characterization of Rabbit Aorta Contracting Substance (RCS) from Guinea Pig Lung and Human Platelets

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Received 20 December 1974

Abstract

SVENSSON J M HAMBERG and B SAMUELSSON *Prostaglandin endoperoxides IX Characterization of rabbit aorta contracting substance (RCS) from guinea pig lung and human platelets* Acta physiol scand 1975 94 222–228

Material causing contraction of the isolated rabbit aorta (rabbit aorta contracting substance RCS) was rel ased from guinea pig lung following perfusion with arach donic acid and from human blood platelets after addition of thrombin to induce aggregation Prostaglandin endoperoxides (prostaglandins G_2 and H_2) were found both in the perfusate of guinea pig lung (1 3 ng ml) and in the medium collected after platelet aggregation (13 37 ng ml) The contractile respons of the isolated rabbit aorta to the pure prostaglandins G_2 and H_2 was also determined These data combined with the quantitative analyses of the endoperoxides released from the lungs and platelets showed that only a minor part of the rabbit aorta contracting activity was due to the prostaglandin endoperox des The major part of the activity consisted of ery unstable material The half lif of this material was about 30 s at 37 whereas at this temperature the prostaglandin endoperoxides had a half life of about 5 min

The release of rabbit aorta contracting substance (RCS) from guinea pig lung following perfusion with bradykinin and slow reacting substance of anaphylaxis (SRS A) as well as by perfusion of sensitized lungs with antigen was described by Piper and Vane in 1969 Prostaglandins E and F were also detected in the perfusate RCS could be distinguished from these prostaglandins by its strong activity on the rabbit aorta and by its instability Vargaftig and Dao later found that also slow reacting substance C (SRS C) and arachidonic acid caused release of RCS from guinea pig lung Formation of a rabbit aorta contracting substance during platelet aggregation induced by collagen and arachidonic acid was recently reported by Vargaftig and Zirinis

The finding that aspirin and indomethacin inhibitors of prostaglandin biosynthesis blocked the release of RCS from guinea pig lung indicated a relationship between RCS and the prostaglandins Gryglewski and Vane suggested that the endoperoxide postulated in the enzymatic conversion of certain poly unsaturated fatty acids into prostaglandins might be identical with RCS Hamberg and Samuelsson recently isolated a prostaglandin

endoperoxide intermediate which was more active than PGE_2 on the isolated rabbit aorta An additional endoperoxide isolated later also had strong activity on the rabbit aorta strip However the prostaglandin endoperoxides were more stable in aqueous medium than RCS indicating that the latter factor was different from the endoperoxides Further studies on the nature of rabbit aorta contracting substance released from guinea pig lung and human platelets are described in the present paper

Materials

Arachidonic acid purchased from Sigma Chemical Co (purity approx 99) was purified by silicic acid chromatography and stored in benzene under argon at −20

Prostaglandins G_2 and H_2 were prepared by incubation of arachidonic acid with sheep vesicular gland microsomes in the presenc of p-mercuribenzoate (5)

Thrombin (Topostasin® Hoffmann La Roche Co) was used to induce platelet aggregat on

Methods

Rabbit a rta st ip

Spirally cut strips 30–40 mm long 3–4 mm wide of thoracal aortas from male rabbits weighing 1 700–2 700 g were suspended and superfused (*cf* 8) with ml/min of Krebs Hensele t b carbonate med um (6) gassed with 93 5 O + 6 5 CO_2 The medium was supplemented with 0 2 glucose and the following antagonists mepyramine maleate (10⁻ g/ml) hyoscine bromide (10^{-7} g/ml) propranolol hydrochloride (2 10 ⁶ g/ml) phenoxybenzamine hydrochloride (10⁻ g/ml) and methysergide bim l ate (10⁻ g/ml) The lower end of each strip was attached to a glass rod and the upper end connected to a Grass force displac ment transducer FT 03C connected to a Grass model 79C Polygraph The strips were stretched by a force of 0 5 ponds and allowed to equilibrate for about 1 h Before each e periment the strips were tested with Hypertensin® (C ba Geigy AG B sl Switzerland) (2 5 μg/ml) and with prostaglandins G_2 and H (100–200 ng/ml)

Perf ed g ea p g lung

Gu nea p g lungs were removed from animals of both se es we gh ng 500– 00 g The lungs were cannulated through the pulmonary artery and suspended in a glass chamber (37) Krebs medium was perfused into the pulmonary artery (2 ml min) and the effluent allowed to superfuse the aorta strip Arachidonic acid (30 μg) was injected into the cannulated pulmonary artery In other experiments th lung was perfused with Krebs medium containing 10 μg ml of arachidonic acid

Hom ge at s of gui a p g l ng

Gui ea pig lungs (2–4 g) were m nced in 5 10 ml of ice-cold 0 1 M potassium phosphate buffer pH 7 4 and homog nized for 2 min w th a Potte El ehjem homog ni er Centr fugation of the homogen te at 8 000 *g* for 1? min ga e a upernatant (mainly m crosomal and soluble fract ons) that was used for the incubat ons

H m n plat lets

Blood from healthy donors who had ot t ken sp r n fo t le st 1 week was col ected w th 7 5 (/) of 0 077 M sod m EDTA and centrifuged at 00 *g* for 15 min The plat let ri h pl sm thu obta ned was centr fuged t 650 *g* f r 15 min The platelet pellet f om l ml of platel t rich plasma was su pended in 5 ml of the following med m 0 15 M NaCl–0 15 M tris HCl buffer pH 7 4–0 077 M od m EDTA (90 8 v/) and subs quently recentr fug d The pell t of washed platel ts was pe ded in 4 ml of Krebs med um not contai ng calcium Platel t agg egat o was monitored with a Chronolog aggrego meter Suspensions of 3 ml of washed plat lets were st rred at 37 fo min and ubseq e tly t eated with 15 U *of thromb n 30 s after the addit on of thromb n the medium containing pl t let aggregates* was f lt red with gentl suct o nd the f ltrate was superfused on the aorta strip for assay of RCS acti ity For d t rminat on of prostaglandin endoperox des 2 samples (1 ml) of the f l rate were coll cted and

added to 15 ml of ethanol and to 15 ml of ethanol containing 75 mg of stannous chloride respectively PGF_2 was determined in these samples as described below

Quantitative determination of prostaglandin endoperoxides

Prostaglandin endoperoxides were determined in perfusate from guinea pig lung and in medium obtained after platelet aggregation by multiple ion analysis using [3 3 4 4 $^2H_4$]$PGF_{2\alpha}$ as internal standard (cf 5) To 1 ml (platelet medium) or 6 ml (lung perfusate) were added immediately either 15 volumes of ethanol or 15 volumes of ethanol containing 0 5 $SnCl_2$ After 2 min at room temperature 5 µg of deuterated PGF_2 were added and the solution extracted with diethyl ether The residue obtained after evaporation was esterified and treated with 50 µl of acetic anhydride and 50 µl of pyridine for 18 h at room temperature The reagent was evaporated *in vacuo* and the residue chromatographed on a column of 0 1 g of silicic acid Elution was performed with 4 ml of diethyl ether light petroleum (? 8 v/v) followed by . ml of diethyl ether light petroleum (5 5 v v) The latter eluate was taken to dryness and subjected to multiple ion analysis The ions at m e 314 (unlabeled derivative) and 318 (d -derivative) (M 3 60 elimination of 3 CH_3COOH) were used

Results

Formation of rabbit aorta contracting substance in perfused guinea pig lung

Injection of 30 µg of arachidonic acid into the cannulated pulmonary artery of perfused guinea pig lung was followed by a strong contraction of the superfused rabbit aorta strip (Fig 1) A prolonged contraction occurred following continuous infusion of 10 µg/ml of arachidonic acid into the lung No or only small response was seen when arachidonic acid was directly allowed to superfuse the aorta strip Arachidonic acid (30 µg) injected following infusion of aspirin (70 µg/ml) or 5 8 11 14-eicosatetraynoic acid (30 µg/ml) gave only weak contraction of the aorta strip

The smooth muscle stimulating material was very unstable By collecting perfusate of guinea pig lung injected with 30 µg of arachidonic acid and incubating the perfusate at 37 for various times prior to test on the aorta strip a series of smooth muscle responses (mm maximum pen deflection) and times of incubation at 37 (seconds) were obtained These tests were performed in random order to avoid possible sensitizing and potentiating effects on the aorta strip Plot of the smooth muscle responses *vs* time revealed a linear relationship This was interpreted to mean that two logarithmic processes were involved *i e* first order decay of the smooth muscle stimulating material and a linear *log*concentration response relationship of the aorta strip In order to determine the slope of the linear part of the *log*concentration response curve (k) perfusates of guinea pig lung injected with arachidonic acid were tested either directly or after rapid dilution with known volumes of Krebs medium By inserting the k value so obtained in the equation *log*concentration = k response the concentrations of RCS (arbitrary units) at different times of incubation



Fig 1 Contractile responses of isolated rabbit aorta exposed to effluent (10 drops during 15 s) from guinea pig lung injected with 30 µg of arachidonic acid A effluent not incubated B effluent incubated at 37 for 30 s C effluent incubated at 37 for 60 s D effluent incubated at 37 for 90 s

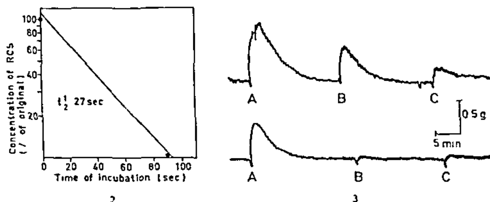


Fig. 2 Decay of rabbit aorta contracting activity in aqueous medium at 37

Fig 3 Contractile responses of isolated rabbit aorta exposed to medium (10 drops during 15 s) collected after platelet aggregation *Upper curve* A without inhibitor B 20 µg/ml of aspirin C 40 µg/ml of aspirin *Lower curve* A without inhibitor B 18 µg/ml of indomethacin C 0 7 µg/ml of indomethacin

could be calculated from the smooth muscle responses The decay of RCS followed first order kinetics and had a half life time equal to 29 ±9 sec (n = 4) (Fig 2)

Formation of prostaglandin endoperoxides in perfused guinea pig lung

Arachidonic acid was continuously infused (10 µg/ml) into the cannulated pulmonary artery When RCS appeared in the perfusate as indicated by contraction of the aorta strip the strip was put aside and 2 samples of perfusate were collected Sample no 3 (6 ml) (Table I) was collected in a flask containing ethanol and sample no 4 (6 ml) in a flask containing ethanol – 0 5 % $SnCl_2$ The flasks were switched with short time intervals during collection in order to compensate for possible variations in concentration of the endoperoxides in the perfusate Samples no 1 and 2 were also collected in flasks containing ethanol without and with $SnCl_2$ respectively In this case no arachidonic acid was infused into the lung The results of quantitative determination of $PGF_{2\alpha}$ in the samples are given in Table I As seen before arachidonic acid infusion low and variable amounts of $PGF_{2\alpha}$ were found A higher level was seen after infusion of arachidonic acid (sample no 3) A pronounced increase was noted in sample no 4 corresponding to infusion of arachidonic acid and SnCl reduction The difference in the amount of $PGF_{2\alpha}$ present in samples no 4 and 3 *reflected the level of prostaglandin endoperoxides G or H or both (1-3 ng/ml)*

In order to test whether the measured levels of endoperoxides could be responsible for the aorta stimulating activity of the perfusate the effect on the aorta strip of PGG_2 and PGH_2 (1-3 ng/ml) was tested No response was obtained

Formation of rabbit aorta contracting substance in homogenates of guinea pig lung

Half a ml of the 8 000 g supernatant of a homogenate of guinea pig lung was incubated at 37 with 2-15 µg of arachidonic acid for 30 s and subsequently diluted with 4 ml of Krebs medium and allowed to superfuse the aorta strip (10 drops during 15 s) A strong contrac-

TABLE I Release of prostaglandin endoperoxides from guinea pig lung.

Exp no	Condition	$PGF_{2\alpha}$ (ng ml)
I	1	0 6
	2	1 0
	3	1 5
	4	3 4
II	1	0 1
	2	0 3
	3	1 4
	4	2 9
III	1	0
	2	1 4
	3	1 4
	4	4 7

[a] 1 No arachidonic acid no reduction 2 No arachidonic acid reduction 3 Arachidonic acid no reduction 4 Arachidonic acid reduction

tion was noted When arachidonic acid was omitted a weak response was recorded This response was elicited also when the diluted supernatant was kept at 37 for 5–10 min The response corresponding to arachidonic acid plus supernatant however was due to unstable material since it disappeared when the diluted incubation mixture was kept at 37 for 5 min The half life time was not determined but appeared to be close to that of RCS formed in guinea pig lung and by platelets

Formation of rabbit aorta contracting substance in platelets

Three ml suspensions of washed human platelets at 37 were treated with 15 U of thrombin After 30 s the aggregated platelets were removed by filtration and the filtrate was assayed

TABLE II Release of prostaglandin endoperoxides from platelets

Exp no	Condition	Time after aggregation (min)	PGF_2 (ng ml)
I	1	5	10
	2	5	8
II	1	1 5	1
	2	1 5	5
III	1	1 5	14
	2	1 5	37
	1	10 0	16
	2	10 0	19
IV	1	0	16
	2	0	53
V	1	2 0	18
	2	0	44
VI	1	0	19
	2	2 0	41

[a] 1 No reduction 2, Reduction

(10 drops during 15 s) on the aorta strip As seen from Fig 3 a strong contraction was recorded This was seen only when the platelet medium was tested a short time (0 5–1 5 min) after the aggregation period Samples kept at 37 for 5 min or more gave no or insignificant response The half life in the first order decay of RCS from platelets was determined to 34±7 s (n = 6) using the method described above for RCS from guinea pig lung When the platelet suspension was preincubated with aspirin or indomethacin formation of RCS was strongly inhibited (Fig 3) The ID , dose of aspirin was about 25 μg/ml

Formation of prostaglandin endoperoxides in platelets

Three ml suspensions of washed human platelets (250 000–500 000 platelets/μl) in Krebs medium were stirred at 37 for 2 min and subsequently treated with 15 U of thrombin Aggregation was allowed to proceed for 30 s The platelet aggregates were removed by filtration and 1 ml of medium was added to 15 ml of ethanol and to 15 ml of ethanol containing 75 mg $SnCl_2$ Table II shows the content of PGF ₂ in these samples The difference between the amounts in the reduced and non reduced samples reflected the level of prostaglandin endoperoxides G_2 and/or H (13–37 ng/ml) PGG and PGH in these concentrations gave very weak response on the aorta strip and could thus not explain the strong rabbit aorta contracting activity of the platelet medium

Discussion

In the present work the formation of material(s) with stimulating effect on the rabbit aorta (RCS) was studied in three systems i e guinea pig lung perfused with arachidonic acid a cell free preparation of guinea pig lung and human platelets treated with thrombin Formation of RCS in the three systems was inhibited by pretreatment with aspirin indomethacin and 5 8 11 14-eicosatetraynoic acid These drugs also inhibit prostaglandin biosynthesis (9 1) The disappearance of rabbit aorta contracting activity produced by guinea p g lung and platelets followed first order kinetics The half life time of the activity from guinea pig lung was 29±9 s and that from platelets 34±7 s In a recent study RCS from guinea pig lung was reported to have a half life of 1–2 min (7) Attempts to recover the smooth muscle stimulating activity by rapid extraction of the acidified aqueous phase with diethyl ether (cf ref 4 and 5) were unsuccessful

Prostaglandin endoperoxides (PGG₂ and/or PGH) were determined in the perfusate from guinea pig lung and in platelet medium recovered after aggregation induced by thrombin As shown by Table I 1–3 ng/ml of PGG and/or PGH were present in the effluent from guinea pig lung perfused with 10 μg/ml of arachidonic acid Medium collected after platelet aggregation contained 13–37 ng/ml of PGG and/or PGH Prostaglandin endoperoxides when tested in these concentrations on the rabbit aorta strip gave no or very weak response This indicated that at least the major part of the rabbit aorta contracting activity formed in guinea pig lung and platelets was due to material which was not identical with the prostaglandin endoperoxides This was also indicated by the fact that the half life of the endoperoxides in aqueous medium (about 5 min ref 5) was much longer than that of the rabbit aorta contracting activity In a separate experiment PGG and PGH_2 were

to perfusate of guinea pig lung and the half life time determined The values obtained did not differ significantly from those obtained with buffer or water excluding the possibility that an endoperoxide degrading activity was present in the perfusate

The present study shows that RCS of P per and Vane is a mixture of at least two components with rabbit aorta contracting activity *I e* prostaglandin endoperoxides and an unstable rabbit aorta contracting factor Further work is needed to cl rify the chemical nature of the latter factor

This work was supported by the Swedish Medical Research Council (proj no 03X 217)

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Acta physiol scand 1975 94 229–235

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Circulatory and Respiratory Effects Evoked by Hypertonic Ventriculo-Cisternal Perfusion

By

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Received 27 December 1974

Abstract

MELLANDER S and J HILLMAN *Circulatory and respiratory effects evoked by hypertonic ventriculo-cisternal perfusion* Acta physiol scand 1975 *94* 229–235

The cerebral ventricular system of anesthetized dogs was perfused with synthetic isotonic CSF and for 80 s intervals with hypertonic CSF of various compositions Hypertonic perfusion evoked centrally mediated marked increases in arterial blood pressure heart rate respiratory rate and ventilation and after some delay an excitatory reaction resembling arousal The responses were coordinated in time with the induced CSF hypertonicity graded in relation to its magnitude and reversible on return to isotonicity The effects which seemed to be elicited from periventricular structures in the brain stem were more pronounced and consistent when CSF hypertonicity was produced by adding NaCl or Na lactate than monosachardes to the isotonic CSF solution Analysis of the cardiovascular responses indicated that they were caused by increased sympathetic vasoconstrictor and cardiac accelerance fibre activity and by inhibition of vagal discharge to the heart The described pattern of response much resembles that evoked by physical exercise a state which might lead to osmolar changes in the brain and CSF of a similar kind to that in the present study as a consequence of the pronounced work induced arterial hyperosmolality It is suggested that such an osmotic mechanism might constitute a metabolic link in the centrally mediated circulatory and respiratory adjustments in exercise

Recent studies from this laboratory have shown that osmotic mechanisms play several important roles in the control of the circulation Thus tissue hyperosmolality resulting from increased cell metabolism is a factor which contributes significantly to the functional hyperemia in exercising skeletal muscle (Mellander *et al* 1967 Mellander and Lundvall 1971 Lundvall 1972) and in active gland (Lundvall and Holmberg 1974) Tissue hyperosmolality is also mainly responsible for the rapid accumulation of plasma fluid which occurs in skeletal muscle during work (Mellander *et al* 1967 Lundvall 1972) Further there is an important osmotic regulation of plasma volume in exercise and in hemorrhage effected by pronounced arterial hyperosmolality which leads to fluid withdrawal from tissues to the blood stream (Lundvall *et al* 1972 Jarhult 1973) In exercise this arterial hyperosmolality is caused by an overflow of osmols from the active muscles (Lundvall *et al* 1972) and in hemorrhage it is caused by glucose release from the liver (Jarhult 1975)

There is also some evidence that arte ial hyp rosmolality of the magnitude occurring in exercise or bleeding can lead to increased sympathetic nervous activity (e g Järhult Hillman and Mellander 1975) This effect might to some extent have been caused by various reflex adjustments from peripheral receptor sites (e g Lasser *et al* 1960 Raizner *et al* 1973) but, hypothetically it might also have been due to an osmotic effect on the central nervous syst m Blood borne hype osmolality would cause o motic dehydration of the brain and the resulting cerebral hypertonicity mig it influence neuronal activity in central nervous autonomic structures In the present stuoy an attempt was made to investigate this hypothesis by studying circulatory respiratory and some other functions during selective cerebral hypertonicity as p oduced by hypertonic ventriculo-cisternal perfusion in the anesthetized dog The p esent paper describes effects observed during short term hypertonic perfusion

Methods

Experiments were performed on 16 mongrel dogs b wt 8 10 kg anesthetized i v with α-chloralose (50 mg kg) and urethane (100 mg kg) The animals were provided with a tracheal cannula and breathed spon taneously through a low resistance two-way valve with negl gible deadspace The expiration tube was connected to a spirometer system for continuous recordings of respiratory rate tidal volume and cumula tive pulmonary ventilation Arterial pulsatile and mean pressures were monitored from a heparinized catheter in the right femoral artery and recorded with Statham transducers Herat rate was recorded with a Grass tachograph using pulse pressure as the trigger In 3 animals calf blood flow was recorded by in serting an optical drop recorder in the popl teal artery after heparinization All these parameters were recorded on a Grass polygraph EEG was registered in two experiments on an Elema recorder using elec trodes fixed at the surface of the skull

An 18 gauge needl with a lateral opening 1 mm from its closed tip was inplanted in the left la eral ventricle and kept in position with dental cement appl ed to the small hole drilled in the skull This cannula as connected a a fine bore polyethylene tubing to a constant infusion apparatus and used f r infusions rtificial cerebrospinal fluid (CSF) In two animals a cannula was also inplanted in the third ventricle o 8 gauge needles were introduced into the cisterna magna through the atlanto-occipetal membrane ne be ng used for CSF drainage and the other for intr cranial pressure measurements with a Statham ransdu r Plac men of the cannulas was checked by the occurrence of a spontaneous drainage of CSF and of resp ratory CSF oscillations and at the end of the experiment by post mortem analysis of the ventri cular system after d e n ections

The synth n CSF was prepared according to Pappenheimer *et al* (196) and had the following compos t on (n ke H O) Na 148 K 3 0 Ca 2 3 Mg 1 6 Cl 131 HCO 27 inorganic P 0 5 glucose 3 0 The synthetic CSF ade hypertonic (400 1 00 mosm kg H_2O) by the addition of sodium cloride sodium lactate gl mannitol The pH of the artificial CSF-solutions was adjusted to 7 40 b bubbling with CO or N_2 pr the fusion and temperature was kept at 37 C Osmolality was determined by thermistor cryoscop d Instruments 31 LAS) In Results spread of data is expressed as the standard error of the

Results

After completion of the p eparation the animals rested in the supine right side position Arterial blood pressure hea t rate cumulative pulmonary ventilation respiratory rate tidal volume and CSF pr s ure were recorded continuously throughout the experiment The CSF draining freely through the open needle in the cisterna magna was collected in consecutive 0 2 ml samples for osmolality determinations Arterial plasma osmolality was determined at intervals CSF p essure measured at the level of the cisternal outflow cannula, was initially 5–10 mm Hg but fell in the steady-state to 1–2 mm Hg after opening of the

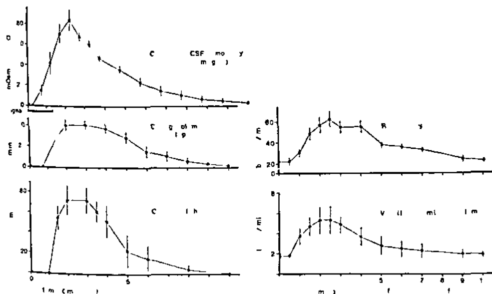


Fig 1 Effects of short term hypertonic ventriculo-cisternal perfusion (signal) on CSF osmolality in the cistern and on mean arterial blood pressure heart rate respiratory rate and cumulative ventilation in anesthetized dogs (n = 8)

drainage Respiratory oscillations of CSF pressure were always present Plasma and CSF osmolality did not differ significantly in the control period the latter osmolality averaging 310±3 mo m/kg H_2O)

After about 20 min of rest the infusion of artificial isotonic CSF through the cannula in the left lateral ventricle was started at a constant rate of 0 55 ml/min CSF drained through the cisternal cannula at the same rate and CSF pressure was therefore unchang d No significant circulatory respiratory or other effects were evoked by the isotonic ventriculo cisternal perfusion

In this steady state the isotonic perfusion was changed to a hypertonic perfusion at the same rate (0 55 ml/min) for a period of 80 s after which the isotonic perfusion was again installed Fig 1 shows for one group of experiments on 8 dog the average changes of CSF osmolality as measured in the cisternal fluid and the alterations of mean blood pressure heart rate and respiration evoked by such short term hypertonic perfusions In the control p riod before the hypertonic infusion mean arterial pressure averaged 126±4 mm Hg heart rate 94±8 beats/min respiratory rate 21±3 breaths/min and ventilation 1 76±0 12 l/min In these experiments the osmolality of the hypertonic artificial CSF was about 800 mosm/kg H O and the CSF hypertonicity was produced by the addition of sodium salts to the isotonic CSF Na lactate in 6 experiments and NaCl in 2 expts The effects were quite similar for both these agents Zero time on the abscissa refers to the moment the hyper tonic CSF reached the left lateral ventricle It can be seen that the hypertonic infusion (signal) followed by isotonic perfusion caused a transient rise of CSF osmolality which in the cistern reached a peak value after some 2 min of about 80 mosm/kg H O above the con trol level The osmolality then gradually returned to the control level in the following 8 m n

It can also be seen that the hypertonic infusion elicited pronounced increases in blood pressure heart rate respiratory rate and cumulative ventilation effects which were quite closely coordinated in time with the CSF hypertonicity The blood pressure and respiratory responses usually commenced some 15 s before the tachycardia response and during this initial period there were signs of a slight bradycardia (not indicated in the figure) Maximal responses were reached after about 2 min Mean arterial pressure at this time was increased by about 40 mm Hg, heart rate by about 70 beats/min respiratory rate by about 40 breaths/min and pulmonary ventilation by about 4 l/min tidal volume increased initially but declined again when the tachypnea became marked All these effects were reversible and were abolished at the time CSF osmolality returned to the control level CSF pressure was not affected by the hypertonic infusion

The blood pressure rise during hypertonic perfusion seemed to be caused at least partly by an increase in peripheral resistance mediated by the vasoconstrictor fibres as evidenced in 3 dogs by a concomitant marked constriction of the resistance vessels in the lower leg region an effect which was absent after regional α adrenoceptor blockade The tachycardia seemed to result from increased adrenergic discharge as well as decreased vagal discharge to the heart as evidenced by a much lessened but not abolished response after atropinization (1 mg/kg b wt i v) or cervical vagotomy The cardiovascular effects were still present after the respiratory responses were blocked by gallamine (3 mg/kg b wt i v) in the artificially ventilated dog

The magnitudes of the blood pressure rise the tachycardia and respiratory responses were graded in relation to the degree of the induced CSF hypertonicity as found during infusions of CSF with both lower and higher tonicity than that in the experiments included in Fig 1 Clearly discernible effects were noted when cisternal CSF osmolality during the peak (cf Fig 1) exceeded the control value by 10 15 mosm/kg H_2O

Besides the described circulatory and respiratory effects hypertonic ventriculo-cisternal perfusion led to an excitatory response in the anesthetized dog which in some respects resembled arousal In some experiments like those included in Fig 1 the excitation was relatively weak and characterized by slight movements of the head and the extremities some increase of skeletal muscle tone semi opened eyelids and somewhat increased responsiveness to reflex stimuli This pattern of response which was preceded in time by the circulatory and respiratory effects disappeared as CSF tonicity returned to normal In some other experiments in particular during higher CSF hypertonicity the excitatory response was much more pronounced The dog then appeared to be clearly alerted tended to raise its head and lick its mouth and made distinct movements of the extremities muscle tone was significantly increased urination and defecation frequently occurred and the animal reacted clearly to various stimuli such as noise When these effects developed (usually about 3 min after the start of the hypertonic infusion) there was a secondary reinforcement of the circulatory and especially of the respiratory effects in one dog ventilation increased to 16 l/min (about 8 times the control value)

Naturally it was considered necessary to administer more anesthetics (chloralose urethane or pentobarbital sodium) in these experiments and this was done routinely as soon as there were any signs of marked arousal The excitatory response then quickly disappeared

but a repeated hypertonic ventriculo-cisternal infusion in such deeply anesthetized animals again evoked the described circulatory respiratory and arousal effects The total amount of anesthetics which the dog received, and survived, in such repetitive infusion experiments sometimes must clearly have exceeded the normally lethal dose

The circulatory and respiratory responses to hypertonic perfusion could be evoked in dogs in which all surgical wound areas were carefully infiltrated with a local anesthetic agent (Xylocain Astra) prior to the infusion indicating that the effects were not caused by pain sensation during arousal

A few preliminary experiments showed that during moderate hypertonicity the circulatory and respiratory effects could be elicited in the absence of any significant concomitant changes in the EEG pattern This finding and the fact that the arousal developed later than the circulatory and respiratory effects suggest that the latter were not initiated by the excitatory response if moderate as in the experiments included in Fig 1

Ventriculo-cisternal perfusion with artificial CSF made hypertonic by the addition of glucose or mannitol to the isotonic solution could evoke a similar pattern of response to that described for sodium salts but the effects were more variable and for a given osmolality increase quantitatively less pronounced than those observed with increased sodium con centration

In two experiments attempts were made to expose only the third and left lateral ventricle to hypertonic perfusion For this purpose an extra needle was inplanted in the third ventricle and was used as the inflow cannula during the perfusion The cisternal cannula was then closed and the infused fluid was drained through the cannula in the left lateral ventricle A shift from isotonic to hypertonic perfusion of the third and lateral ventricle in these ex periments caused no significant changes of blood pressure heart rate respiration, or alert ness" The typical pattern of response quickly developed however upon diversion of the hypertonic infusion to the distal sections of the ventricular system which was accomplished by opening the cisternal outflow cannula and closing the drainage from the left lateral ventricle It thus appears from these preliminary experiments that the structures sensitive to hypertonicity and responsible for the described effects might be localized in periventricular areas of the brain stem

The pH of cisternal CSF was unaltered or occasionally slightly increased during the hypertonic ventriculo-cisternal perfusion the latter effect probably being the result of the evoked hyperventilation Arterial plasma osmolality was not changed by the hypertonic ventriculo-cisternal perfusion

Intravenous infusion of hypertonic mannitol which in the steady state raised arterial plasma osmolality by about 40 mosm/kg H_2O (2 expts) led after a delay of 1 2 min to an increase of CSF osmolality in the cisterna magna After 5 min the osmolality increase in the cistern was about 70% of that in plasma

Discussion

This study showed that hypertonic ventriculo-cisternal perfusion in the anesthetized dog evoked centrally mediated increases in arterial blood pressure heart rate and respiration

and after some delay an excitatory reaction which resembled arousal The responses were coordinated in time with the CSF hypertonicity graded in relation to its magnitude and reversible upon return to isotonicity The effects were more pronounced and consistent when the synthetic CSF perfusate was made hypertonic by the addition of sodium salts than of glucose or mannitol to the isotonic solution Analysis of the cardiovascular effects indicated that they were caused by increased sympathetic vasoconstrictor and cardiac accelerance fibre activity and by inhibition of vagal discharge to the heart Results obtained after sympathectomy suggested that humoral agents such as vasopressin did not contribute significantly to the peripheral vasoconstriction

The effects might at least partly be interpreted to be mediated by a Na sensitive mechanism similar to that described by Andersson *et al* (1972) for the control of fluid balance and blood pressure The Na sensitive system for this control was reported to be localized in the vicinity of the third ventricle whereas the effects described in the present study seemed to be evoked mainly from periventricular structures in the brain stem The exact site of action however remains to be determined

It appears that the present observations might be taken to indicate a physiologic role of osmotic mechanisms in the central control of the circulation, respiration and perhaps also of alertness This might for instance apply to the situation of physical exercise in which a pronounced arterial hyperosmolality rapidly develops (Lundvall *et al* 1972) as a result of water flux into and release of metabolic products from the contracting striated muscle fibres (Lundvall 1972) The arterial hyperosmolality mainly due to raised plasma sodium and lactate concentrations in turn would cause secondary passive dehydration of the brain and intracerebral hypertonicity due to preferential water transfer across the blood brain rrier CSF tonicity would also increase as shown for instance by the present intravenous pertonic infusion experiments In absolute figures the Na and Cl concentrations ould seem to be increased to the greatest extent (*cf* Milhorat *et al* 1971) a situation thus resembling, qualitatively the present hypertonic ventriculo-cisternal perfusions with raised NaCl concentration in the perfusate The abovementioned hypothesis is strengthened by the fact that the cardiovascular respiratory and autonomic nervous effects and perhaps also the arousal evoked by hypertonic ventriculo-cisternal perfusion so much resemble the adjustments caused by exercise *per se* Further Holland *et al* (1959) found that intra-carotid injections of hypertonic solutions evoked a pattern of response which in several respects resembled the one found in this study The mentioned osmotic mechanism then might be contributing to the centrally mediated circulatory and respiratory adjustments in work that are believed to be metabolically linked it may thus be partly responsible for the gradually developing circulatory and respiratory changes during exercise and for the maintenance of such effects in the postexercise period It is possible that an osmotic mechanism also might contribute to centrally mediated adjustments occurring in other hyperosmolar states *e g* in severe hemorrhage (*cf* Järhult 1973)

This study was supported by gr t B75 14X 210-09A from the Swedish Medical Research Council and by a grant from the Medical Faculty Lund

Note added in proof After this paper was submitted for publication an interesting article by H Schad and H Seller has appeared (Pflug. Arch 1975 353 107 121) which demonstrates a distinct influence of intracranial osmotic stimulation r nal sympathetic activity

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Acta physiol scand 1975 94 236–243
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Effect of Insulin on Albumin Production and Incorporation of ^{14}C-leucine into Proteins in Isolated Parenchymal Liver Cells from Normal Rats

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Received 31 December 1974

Abstract

DICH J and C N GLUUD *Effect of insulin on albumin production and incorporation of ^{14}C leucine into proteins in isolated parenchymal liver cells from normal rats* Acta physiol scand 1975 94 236–243

Parenchymal rat liver cells were isolated by a modification of the collagenase method of Quistorff Bondesen and Grunnet The cells secreted albumin into the medium and incorporated ^{14}C leucine both into cell proteins and proteins secreted into the medium Albumin production measured from the immunologically determined increment in the incubation medium was 1 7±0 ? μg albumin/min per g liver wet wt This is about 30 of the rate of production in the perfused liver Addition of insulin (10^{-4}–10 M) enhanced albumin production (50 17) and incorporation of ^{14}C leucine both into albumin (50–8) secreted proteins (40 9) and cell proteins (0 8) Insulin does not increase the production of albumin by depleting the cells The effect of insulin on albumin production is compatible with an effect on the rate of synthesis as the specific activity of albumin is unaffected by addition of insulin

Observations *in vivo* that uncontrolled diabetes is accompanied by loss of body weight depletion of tissue protein negative nitrogen balance and lowered concentration of plasma albumin has led to the acceptance of the idea that insulin plays a regulatory role in protein metabolism (Manchester 1970)

Insulin administered *in vitro* to muscle and several other tissues from both normal and diabetic animals enhances the incorporation of labelled amino acids into protein (Manchester 1970) probably indicating an increased rate of protein synthesis Addition of insulin *in vitro* to livers from diabetic animals also enhances the albumin production and the incorporation of labelled amino acids into protein (Marsh 1961 Penhos and Krahl 1962 1963) Addition of insulin to livers from normal animals does not increase albumin production (Marsh 1961 John and Miller 1969) and only in some cases increases the incorporation of labelled amino acids into protein (Penhos and Krahl 1962 1963 Mondon and Mortimore 1967 John and Miller 1969)

In the present investigation isolated parenchymal liver cells from normal rats were used

to study the *in vitro* effect of insulin on albumin production and the incorporation of ^{14}C leucine into secreted proteins (*i e* proteins found in the medium) and cell proteins These cells have earlier been shown to synthetize albumin (Weigand *et al* 1971 East Louis and Hoffenberg 1973) and to be sensitive to insulin (Johnson *et al* 1972 Zahlten Stratman and Lardy 1973)

Materials and Methods

A mals Female Wist r rats weighing approximately .00 g were used Th animals were fed ad libitum on a laboratory chow

Prepa at on of l cells Cells were prepared according to the method described by Quistorff *et al* (1973) with the following modifications Hyaluronidase was omitted in the perfusion med um and the concentration of collagenase was 0 05% Further the enzyme med um was perfused through the li er for 25 min the flow rate being 7 ml min The yi ld of cells was ? 5–3 5 ml of tightly packed cells from a 200 g rat

Incubat on The incubation medium consisted of Hanks solution (Hanks and Wallace 1949) containing 15 mM glucose 10 mM HEPES amino a ids as in rat plasma (Scharff and Wool 1964) and 1 bovine serum albumin pH was initially 7 4 and decreased at the end of the experiments to 7.2–7 3

The li er cells were incubated in Warburg flasks at 37 C w th atmospheric a r as the gas phase Carbon dioxide was absorbed in KOH in the center wells Incubat on volume was 1 5 ml The cell concentration was 0 05–0 10 ml of tightly packed cell per ml cell suspens on (Hepatocrit 5–10 measured as the usual hematocrit) This corresponded to a prote n concentration of 10 0 mg per ml cell suspension 0 5 μCi L (U ^{14}C)-leucine and insulin or sol ent were added after 15 min of p eincubation As insulin *is* known to be degraded very qu ckly in the l er (Field 197) addition of insulin (or sol ent) was repeated after 45 and 75 min of incubation The relevant parameters were measured in the period from 15 to 1 0 min

Album n p od ct o Production of albumin was stopped by chillin on ice Li er cells were immediately se arated from incub tion med um by centrifugation Album n production was determined from the in crement of rat albumin concentrat on in the m dium measured immunologically accord ng to the method of Man ini Cabonara and Heremans (1965) The extracellular olume n the cell suspens on was calculated from the hepatoc it.

In some experiments in which the i tracellular amount of albumin was measured the li er cells in the suspens on were destroyed by ultrasonification before analysis was performed as described abo e

Inco p at on of C leucine to album n The amount of label incorporat d into albumin was deter mined as follows Rat album n was added excess to 00 μl supernatant of the cell suspen ion (prepared as above) All albumin was precipitated with specifi anti rat album n serum Th precipitate was washed 3 times with physiological saline contai ng carrier leuc ne The f nal precipitate was d ssol ed in 500 μl

TABLE I Albumin p oduction (μg per ml cells) and i corporation of C leucine into albumin and secreted p oteins (cpm 10⁻³ per ml cells) Aft 15 min of preincubation C leucine and insul n or solvent were added The incub tion time was 105 m n Figu es a e gi en as means ± S E of 5 expts. each performed in dupl cate The s gnificance of the differen e between means was determined by the paired-data t test

	Control	Insul n (10^{-4} M)	Difference	Insulin (10^{-4} M)	D fference	Insulin ($10^{-?}$ M)	D fference	Insul n (10^{-} M)	Dif e nce
Albumin produ tion	96 ± 30	443 ± 41	147 ± 1 p 0 C01	352 ± 4	63 _ 16 p 0 0	344 ± 6	48 ± 5 p < 0 001	348 ± 34	5 _ 6 p 0 001
I corpo a t on into album n	31 ± 38	34 ± 54	111 ± .3 p 0 01	8 ± 45	51 ± 13 p 0 0	56 ± 4	5 ± 9 p 0 05	47 ± 37	18 ± ? N S
I corpo tion nto secr t d prot	6 3 44	874 ± 68	51 ± 79 p 0 01	747 ± 54	1 4 ± 6 p 0 01	694 ± 47	71 ± 4 p 0 05	6 9 ± 5	56 ± 17 p < 0 05

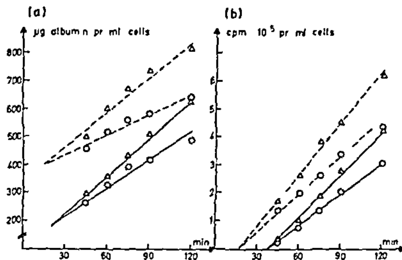


Fig. 1 Tim course of albumin product on (a) and incorporation of ^{14}C leucine into albumin (b) in the cell suspension (–) and in the incubation medium (——) Experiment with addition of insulin (10^{-4} M) is indicated by and control by ○ ^{14}C leucine was added at 15 min

Protosol (New England Nuclear) and counted in a Packard 000 TriCarb liquid scintillation spectrometer Th incorporated activity was expressed as cpm ml cells (*i e* tightly packed cells)

Control experiments were performed to check if the washing procedure avoids contamination of the albumin precipitate with ^{1} C leucin Cell suspensions were preincubated for 15 and 90 min ^{14}C leucine was added and the incubations were stopped immediately Samples were proce sed as above In no case the rad oacti itv in the samples was higher than in the background

In orporation of C le cine into s creted proteins and cell prote ns 100 μl supernatant of the cell sus p nsion or 50 μ l cell suspension were pipetted on discs of Whatman 3 MM chromatography paper and further processed and counted as described by Mans and Novelli (1961) and Schreiber *et al* (1971) From the incorporated radioactivity into secreted proteins (*i e* proteins found in the medium) and into proteins of the cell suspension the incorporation into c ll proteins was calculated The acti ity was expressed as cpm ml c lls

Othe anal i al methods Oxygen consumption was measured in a Gilson respirometer and ATP ac cording to Ad m (196)

Chemi als Insulin was a g nerous gift from Novo Industries The hormone was dissol d in d lute hydrochlori acid and neutralized I inal dilution was made with 0 04 M phosphate buffer pH 7 4 con taining 1 album n Other chemicals were of analytical grade

Results

Albumin production

Addition of insulin increased significantly both the immunologically measured albumin production and the incorporation of ^{14}C leucine into albumin (Table I) Insulin (10 ^{4} M) increased both parameters 50 insulin (10 M) 21 insulin (10 M) 16 and 11 % respectively and insulin (10 M) 17% and 8 % respectively The last mentioned result was not significant The effect of insulin correlates well for the 2 independent measured parameters (r 0 96 p 0 01)

The increment both in the medium and in the cell suspension of rat albumin concentra tion proceeded linearly in the period investigated (Fig 1) In control experiments the im-

TABLE II Incorporation (cpm × 10^{-2} per ml cells) of ^{14}C leucine at different times into cell proteins in normal parenchymal rat liver cells Experimental details as in Table I

Incubation min	Control	Insulin (10^{-6} M)	Difference	Insulin (10^{-8} M)	Difference	Insulin (10^{-9} M)	Difference	Insulin (10^{-} M)	Difference
15	739 ± 26	884 ± 32	145 ± 7 $p < 0.001$	8_6 ± 34	87 ± 9 $p < 0.001$	801 ± 39	6? ± 17 $p < 0.05$	799 ± 37	60 ± [illegible] N S
45	1 501 ± 77	18 4 ± 83	323 ± 36 $p < 0.001$	1 668 ± 86	167 ± ?6 $p < 0.01$	1 606 ± 8_	105 ± 8 $p < 0.0_$	1 548 ± 85	47 ± 46 N S
75	1 874 ± 117	2 278 ± 1	3 404 ± 6 $p < 0.001$	2 015 ± 1_7	141 ± 5 $p < 0.01$	1 933 ± 117	59 ± _6 N S	1 883 ± 109	9 ± 37 N S
105	20 0 ± 146	2 539 ± 169	519 ± 44 $p < 0.001$	2 190 ± 140	170 ± 41 $p < 0.0$	2 044 ± 1?_	4 ± 56 N S	[illegible]	[illegible] N S

munologically measured rate of albumin production was 2 8 ± 0 3 μg/ml cells per min (Table I) *Assuming that 0 6 ml packed cells corresponds to 1 g liver wet wt (Bucher et al* 1972) this rate corresponds to 1 7 ± 0 2 μg albumin/g liver wet wt per min which is about 30% of the rate of production in normal perfused rat liver (Hoffenberg Gordon and Black 1971 Dich Hansen and Thieden 1973) but in agreement with earlier observations in isolated liver cells (East Louis and Hoffenberg 1973) The increment of the incorporation of ^{14}C leucine into rat albumin followed a similar time-course as the production of albumin determined immunologically (Fig 1) A time lag period of 15-20 min (transit time) was found before labelled albumin appeared in the medium This is in agreement with earlier findings in liver slices (Judah and Nicholls 1970) and *in vivo* experiments (Peters and Peters 1972)

The intracellular amount of albumin determined from the difference between the amount of albumin in the cell suspension and in the medium was nearly constant in the period investigated (Fig 1) Calculated from three independent experiments the intracellular amount of albumin was 109 ± 1 μg albumin per g liver wet wt which is about 30 of the amount found *in vivo* (Gordon and Humphrey 1961 Peters and Peters 1972) Addition of insulin increased the intracellular amount of albumin with 18

Incorporation into secreted proteins and cell proteins

The incorporation of ^{14}C leucine into proteins secreted into the medium was enhanced in a similar manner as insulin increased the incorporation into albumin (Table I)

The effect of insulin on the incorporation of C leucine into cell proteins was measured 15 45 75 and 105 min after the initial addition of insulin (Table II) Insulin (10 and 10 M) increased the incorporation about _0 and 10 at all times measured Insulin (10 M) gave a significant effect (7) at 15 and 45 min after addition The effect of insulin (10 M) was not significant

Incorporation into proteins secreted into the medium showed a similar time-course as the incorporation into albumin (Fig 1 and 2) Incorporation of ^{14}C leucine into proteins in the cell suspension started immediately after addition of tracer The incorporation pro-

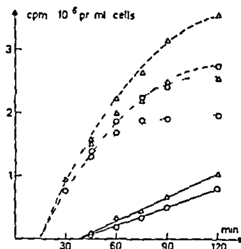


Fig. . Time course of incorporation of [14]C leucin into proteins in the cell suspension (-) proteins secreted into the incubation medium (——) and cell proteins ( ) Experiment with addition of insulin (10^{-8} M) is indicated by and control by ○ [14]C leucine was added at 15 min

ceeded linearly for 60–75 min A plateau was reached after about 90 min (Fig 2) Addition of insulin (10^{-8} M) resulted in a prolonged linearity and a plateau as in the control experiments was not obtained during the incubation time The resulting curves for the incorporation into cell proteins have a similar form as the curves for the cell suspension although the duration of the period of linearity was shorter (Fig 1)

O_2-consumption and content of ATP

This was measured to check the viability of the cell preparations O_2-consumption in control experiments was 1 3 μmol O_2/g liver wet wt per min and declined to 1 0 μmol/g liver wet wt per min at the end of the experiments The content of ATP at the end of the experiments was 1 5 μmol ATP/g liver wet wt These results agree with earlier observations (Quistorff *et al* 1973) and are about 50 % of the results obtained in perfused liver (Dich *et al* 1973)

Discussion

It has earlier been demonstrated in perfusion experiments that albumin production (i e the amount of albumin liberated into the perfusion medium) is decreased in the diabetic liver (Marsh 1961) Addition of insulin to the perfusion medium increased albumin production (Marsh 1961) A similar effect of insulin on perfused liver from normal animals could not be demonstrated (Marsh 1961 John and Miller 1969) The present investigation shows that addition of insulin to parenchymal rat liver cells from normal rats gives rise to both an increased production of albumin and an increased incorporation of [14]C leucine into albumin

Further it has earlier been shown that incorporation of labelled amino acids into proteins is decreased in liver slices (Penhos and Krahl 1962) perfused liver (Green and Miller 1960 Penhos and Krahl 1963) and isolated parenchymal liver cells (Ingebretsen *et al* 1972) from diabetic rats Addition of insulin increased the incorporation (Penhos and Krahl 1962 1963)

Attempts to demonstrate a similar effect on livers from normal rats have given equivocal results With ^{14}C leucine as tracer no effect of insulin could be demonstrated in perfusion experiments (Penhos and Krahl 1963 Mondon and Mortimore 1967) On the contrary Mondon and Mortimore (1967) using ^{14}C valine and John and Miller (1969) using ^{14}C lysine found an increased incorporation when insulin was added to the perfusion medium In the present investigation insulin enhanced the incorporation of ^{14}C leucine into both cell proteins and secreted proteins

The reason for this discrepancy between some of the earlier results concerning both al bumin production and incorporation of labelled amino acids and the present result is not clear Meanwhile methodological differences must be considered Liver slices are not uni form oxygen diffusion to the cells inside the slices is difficult and the content of ATP is low (Thieden 1968 Grunnet *et al* 1973) Perfusion experiments are more physiological but the variation between individual livers is considerable if the liver does not act as its own control In experiments with isolated parenchymal liver cells the results are reproducible the content of ATP is acceptable the cells can act as their own control and they represent only one population of cells

The amount of albumin and the amount of ^{14}C leucine incorporated into albumin in the intracellular pool was nearly constant both in the control and in the insulin experiments (Fig 1) This implies that the increased albumin production and incorporation of ^{14}C leucine into albumin found in the medium could not be due to a depletion of albumin from the cells

The increased incorporation of ^{14}C leucine into protein after addition of insulin could theoretically be due to an increased synthesis and/or an increased specific activity of tracer inside the cell The latter could be caused by an enhanced transport of amino acids into the cell and/or by a decreased degradation of proteins

Provided that the synthesis of albumin and other proteins in the liver cell use amino acids from the same intracellular pool an increased transport of amino acids into the cell could not be the only explanation of our results as insulin did not change the specific activity of albumin Further insulin also increased the albumin production determined immunologically (Table I) In contrast to the present findings Miller and Griffin (1972) found that addition of insulin increased the amount of amino acids intracellularly in the perfused rat liver On the other hand Schreiber and Schreiber (1972) have shown that isolated parenchymal rat liver cells are freely permeable for leucine and methionine

Insulin has earlier been reported to decrease the degradation of protein in the perfused liver (Mondon and Mortimore 1967 Mortimore and Mondon 1970) Addition of insulin (10^{-8} M) increased albumin production by 50% (Table I) If this change should be caused only by a decreased degradation and the considerable rate of protein turnover in the liver ($t/2 = 16-18$ h) is taken into account an increase in the specific activity of albumin in the insulin treated cells would have been expected

The increased albumin production measured immunologically the increased incorpora tion of ^{14}C leucine into albumin and also the unchanged specific activity of albumin are consistent with a direct effect on insulin on the synthesis of albumin In favour of the regu latory role of insulin on protein synthesis are further more the observations that experi

mental diabetes results in a loss of rough endoplasmic reticulum and polysomes (Reaven *et al* 1973) and that administration of insulin to streptozotocin diabetic animals restores the ultrastructure of the liver cell to that seen in normal animal (Pain *et al* 1974) Insulin also stimulated the formation of polyribosomes in cultures of rat liver cells (Gerschenson *et al* 1974) The site of action is probably located on the translational level since addition of actinomycin D does not reduce the effect of insulin on the incorporation of labelled amino acids into proteins (Gerschenson Davidson and Anderson 1974)

The authors thank Ida Cohrt Tonnesen for expert technical assistance Financial support was provided by Fonden til Lægevidenskabens Fremme and F L Smidths fond

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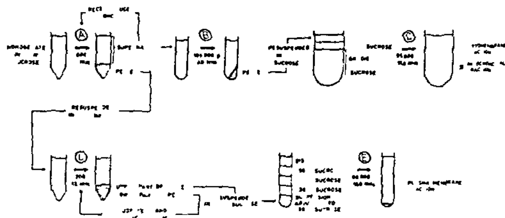


Fig 1 Procedure for the simultaneous isolation of 3 membrane fractions from the rabbit renal med lla Centrifugations A and D were carried out in an MSE Multex centrifuge centrifugations B C and E in a Beckman L 65B ultracentrifuge The following tubes and rotors were used A and D 15 ml con cal centri fuge tubes B rotor 50 Ti C 1 3 5 inch tubes in rotor Sw 27 E 5 9 4 inch tubes in rotor Sw 7 The bands in the gradients were collect d with a syringe

Marker en yme assays

Protein was determined by the method of Lowry *et al* (1951) using bovine serum albumi 1 as a standard *5 nucleotidase* (5 ribonucleotide phosphohydrolase EC 3 1 3 5) was assayed at 37 C with 10 mM adenosine 5 monophosphate (Boehringer Mannheim GmbH West Germany) in a 0 10 M Tris acetate buffer (pH 7 5) containing 10 mM $MgCl_2$ (Widnell 197) Liberated phosphate was determined according to Fiske and SubbaRow (19 5) Assays were also made with 10 mM adenosine 3 monophosphate or β-glycerophosphate as substrate For comparison the above procedure was applied to homogenates of rabbit renal medulla rabbit liver and rat liver *NADH cytochrome c reductase* (NADH cytochrome c oxidoredu tas EC 1 6 99 3) activity was assayed by the method of Mahler (1955) Cytochrome c (Sigma Type IV) was us d at a concentration of 7 μM *Cytoch ome c oxidase* (Ferrocytochrome c oxygen oxidoreductase EC 1 9 3 1) acti ity was determined by the method of Wharton and Tzagoloff (1967) Cytochrome c (Sigma Type IV) as reduced with ascorbate and used at a concentration of 3 μM *Acid phosphata e* (orthophosphoric monoester phosphohydrolase pH 5 EC 3 1 3 ?) was assayed at 37 C in a 0 05 M acetate buffer (pH 5 0) using 25 μM β glycerophosphate as a substrate Inorganic phosphate was determ ned according to Fiske and SubbaRow (19 5)

Assay of p ostagland n synth tase

Arachidonic acid was stored frozen as the methyl ester (Applied Science Lab Pennsylvania USA) Immediately before use 400–900 μg of the ester was hydrolyzed as described elsewhere (Larsson and Ånggård 1974) Tritium labelled arachidon c ac d [5 6 8 9 11 12 14 15 H_8] arachidonic acid (NEN Chemicals GmbH Dreieichenhain West Germany specific acti ity 5–80 Ci/mmol) was added to the non radioacti e substrate before purif cation on a sil ic ac d column The rad ochemi al purity of the tritium lab lled arachidonic acid was checked by thin layer chromatography on silica gel impregnated with s l er nitrate (syst m hexane diethyl ether–acetic acid 70 30 1 by vol) The arachidonic acid was dissolved in a small volume of ethanol which was then mixed with 0 10 M potassium phosphate buffer (pH 7 4) and added to the incubation medium

An amount of homogenate or membrane fraction corresponding to 0 15–2 mg protein was incubated in 0 10 M potassium phosphate buffer (pH 7 4) contain ng 3 mM reduced glutathione 1 mM EDTA 0 1 bovine s rum albumin 16 –60 μM (10–80 μg ml) arach donic ac d 1 5 μCi of [^{3}H]arachidon c acid and 0 0 ml of a supernatant of a boiled 10 homogenate (Samuelsson 1967) of rabbit renal medulla The total incubation volume was 1 0 ml Incubations were carried out at 37 C for 0–1 0 min and terminated by the addition of 9 volumes of abs alcohol The samples were then left at +4 C overnight Double deter

minations were always performed Blanks without active enzyme were simultaneously incubated In the experiments where the distribution of prostaglandin synthetase in different membrane fractions was determined the amount of protein incubated was about 0 5 mg, substrate concentration was 130 μM and incubation time 20 min

After evaporation of the alcohol precipitated samples the aqueous residue was acidified to pH 2–3 with 2% formic acid and extracted twice with 3 volumes of diethyl ether The ether phases were reduced to dryn ss in vacuo The residue was dissolved in 0 5–1 ml ethyl acetat –benzene (1 9 by vol) applied on a 0 5 g silicic acid column (Unisil 100–200 mesh Clarkson Chemical Co Williamsport, Pennsyl ania USA) and eluted in turn with 30 ml of the following 4 sol ents ethyl acetate–benzene (1 9 by vol) ethyl acetate-benzene (8 by vol) ethyl acetate and methanol (Hamberg 1969) A 5 ml aliquot of each eluat was count d in a Beckman LS 155 liquid scintillation counter The scintillator consisted of 4 g of PPO and 0 50 g of POPOP p r liter toluene

Th amount of arachidonic acid converted to prostaglandin l ke substances was calculated from the amount of rad oactivity recovered in the 3 last eluates minus blank alues and the specific acti ity of the incubated arachidonic acid Correction was made for the loss of one tritium atom from C 9 of the precursor dur ng th conv rsion.

Samples of the 4 eluates obtained from the silicic acid column were analyzed by thin layer chromatography on sil ca gel The solvent system was chloroform–methanol–acetic acid–water (90 9 1 0 65 by vol) (Nugteren *et al* 1966) Prostagland n E_2 (PGE$_2$) and prostagland n A_2 (PGA) were ncluded as refere ces The ethyl acetate benzene (1 9) eluate contained the unconverted [^{3}H]arachidonate The ethyl ac tate eluate contained rad oacti e material mainly co-chromatographing with PGE$_2$ The radioactivity in the ethyl acetate benzene (8) and methanol eluates consisted in addition of less polar and more polar compounds resp cti ely

The inh bitory effect of indomethacin on the conv rsion of arachidoni acid to prostaglandin like compounds was checked in samples of the homogenate or fraction II incubated as abo e with a substrate concentrat on of 130 μM and in the presence of indomethacin (Dumex Copenhagen Denmark) The prostaglandin synth tase acti ity was inh bited in both fractions The inhibition was about 30 at 10^{-6} M 70 at 10^{-5} M and 90 at 10^{-4} M indomethacin

Results

Ultrastructure of the isolated membrane fractions

The ultrastructural appearance of the membrane fractions I II and III can be compared on the survey electron micrographs in Fig 2 3 and 6 The membranes of fractions I and II are shown at high magnification in Fig 4 and 5

Fraction I (plasma membrane fraction) This fraction consisted of open-ended membrane flakes or vesicles the profiles of which varied in size from 0 2 to 3 μm (Fig 2) When cut at right angles (Fig 4 a and b) they were seen to have a distinct triple layered appearance and a total thickness of 8–9 nm This corresponds well to the ultrastructure of the plasma membrane of renal cells (Sjostrand 1963) and of plasma membranes isolated from liver cells (Benedetti and Emmelot 1968) Some membranes had an asymmetric triple layered structure (Fig 4 b) This was possibly due to some cytoplasmic material adhering to the inner aspect of the plasma membrane Five layered structures resembling tight junctions were also observed in this fraction Membrane fragments resembling distorted mitochondria were seen only very rarely

Fraction II (cytomembrane fraction) This fraction consisted of closed membrane vesicles with a diameter of 0 03–0 2 μm (Fig 3) Both smooth surfaced and ribosome studded membranes were present When cut at right angles most membranes showed a triple layered structure (Fig 5 a and b) with a thickness of about 6 nm The triple layered structure was

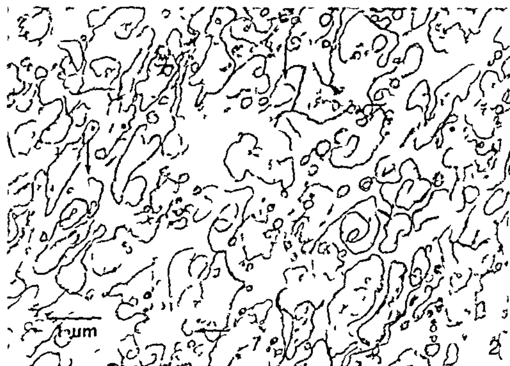


Fig Survey electron micrograph of fraction I (plasma membrane fraction) It consists mainly of membrane sheets often with free ends (arrows) Some small vesicles are also present The size of the membrane profiles is 0 3 μm 13 000

usually less distinct than that seen in the membranes of fraction I The presence of some stained material in the light intermediate layer of the membranes often produced a globular pattern This pattern was also evident in grazing sections parallel to the membrane surface (Fig 5 b) In contrast to fraction I only few membranes had a thickness of 8-9 nm Occasionally membrane limited lyso ome like bodies were seen in this fraction

Fraction III (mitochondrial fraction) This fraction consisted almost exclusively of mitochondria (Fig 6) which often were osmotically affected with a widened outer compartment —intracristal space and a dense matrix Lysosome like bodies and some rough surfaced membrane vesicles were also present

Distribution of enzyme markers in the fractions

The distribution of protein and marker enzymes in the different fractions is seen in Table I and the relative specific activities of the enzymes are shown in Fig 7

Cytochrome reductase activity was very low in fraction I slightly higher in fraction III but increased 2 5 times over the homogenate in fraction II Cytochrome oxidase activity was low in fractions I and II but increased 3 0 times over the homogenate in fraction III

It was not possible to determine in the present experiments the activity of 5 nucleotidase in the fractions Under identical incubation conditions the rate of hydrolysis of adeno-

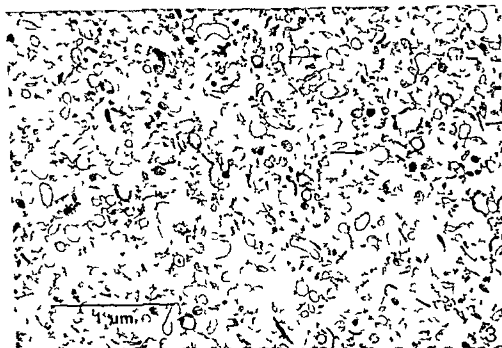


Fig 3 Survey electron micrograph of fraction II (cytomembrane fraction) It consists of small vesicles with a diameter of 0 03–0.2 μm Both smooth surfaced and rough surfaced (arrows) ves cles are present Some dense lysosome-like bodies are also seen × 32 000

sine 3 monophosphate and β glycerophosphate was approximately the same as that of adenosine 5 monophosphate This was in contrast to rat liver homogenates where the rate of hydrolysis of adenosine 5 monophosphate was about 5 fold that of the other substrates

T BLE I D str butio of marker e zyme act it es in membrane fractions from rabb t renal medulla For incubation cond tions see under METHODS The alues are means ± S E. of 3 5 expts

	Homogenate absolute lues	Yield in of homogenate				Reco ery
		I Plasma membrane fraction	II Cyto-membrane fraction	III Mito-chondrial fraction	D scard	
Prote n	270 ± 17 mg	0 17 ± 0 037	4 ± 0 18	5 ± 0 30	84 ± 5	90 7
Cytochrome reductase	13 ± 6 μ mol cytochrome c min	0 03 ± 0 018	6 8 ± 1	9 ± 0.29	6 ± 14	7 ± 1
Cytochrome oxidase	3 7 ± 0 76 μ mol cytochrome c m n	0 058 ± 0 01	1 8 ± 0 39	6 9 ± 1 1	101 ± 19	110 ± 0
Acid phosphatase	8 1 ± 0 34 μ mol P_i l berated m n	0 14 ± 0 051	5 3 ± 0 0	5 4 ± 0 30	73 ± 4	83 ± 5

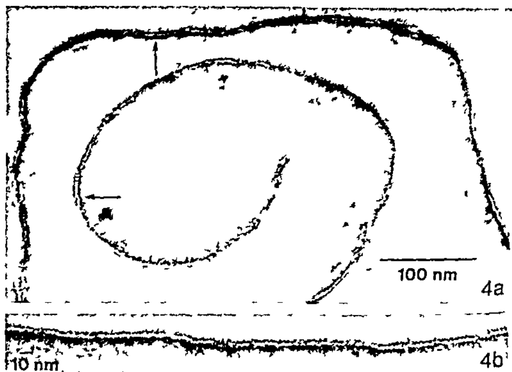


Fig. 4 a Membrane sheet in fraction I (plasma membrane fraction) When cut at right angles the membrane is seen to be distinctly triple layered (arrows) and to have a thickness of 8 9 nm Same magnification as Fig 5 a 240 000

Fig 4 b Membrane in fraction I (plasma membrane fraction) It is distinctly triple layered and asymmetrical Same magnification as Fig 5 b 330 000

Kinetics of the prostaglandin synthetase assay

Effect of substrate concentration As shown in Fig 8 the reaction velocity increased with increasing concentrations of arachidonic acid although the increment in velocity was small for concentrations over 130 μM For this reason an arachidonic acid concentration of 130 μM was used in the incubations

Time course Under the conditions presently employed (Fig 9) the reaction proceeded almost linearly for the first 20–30 min Accordingly an incubation time of 20 min was chosen for the assays Under these conditions less than 3 of the substrate was consumed in each incubation

Proportionality The amount of substrate converted to prostaglandin like compounds was proportional to the amount of membrane fraction incubated (Fig 10) It was found that in order to obtain a sufficient accuracy in the prostaglandin synthetase assay an amount of membrane fraction corresponding to 0 3–0 6 mg protein had to be used for each sample

Distribution of prostaglandin synthetase in the membrane fractions

The homogenate of the rabbit renal medulla converted exogenous arachidonic acid to prostaglandin like products at a rate of about 0 1 nmol arachidonic acid/min per mg protein (Table

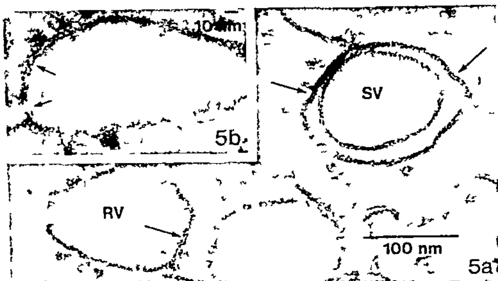


Fig 5 a Smooth-surfaced (SV) and rough-surfaced (RV) vesicles in fraction II (cytomembrane fraction) Both types of membranes are triple laye ed (arrows) and ha e a total th ckness of 6–7 nm Compare the thickness of the membrane in Fig. 4 a 240 000

Fig 5 b Rough-surfaced vesicle in fraction II (the cytomembrane fraction) To the right where the mem brane is cut at r ght angles (arrows) it is seen to be triple layered with a total th ckness of about 6 nm Compare the th ckness of the membrane in Fig. 4 b In the lower part of the p cture where the membrane is cut parallel to its surface a globular pattern is seen × 330 000

II) Prostaglandin synthesis was found in all 3 membrane fractions (Table II) In the plasma membrane (I) and mitochondrial (III) fractions the specific activity of the prostaglandin synthetase was approximately the same as in the homogenate while in the cytomembrane fraction (II) it was 2 4 times that of the homogenate

TABLE II Distribution of prostaglandin synthetase in membrane fractions from rabbit renal medulla Samples of the d fferent fract ons (about 0 5 mg protein from the homogenate and fractions I III and about 0 3 mg protein from the d scard) were ncubated in a 0 10 M phosphate buffer (pH 7 4) at 37 C for 0 min The concentration of arachidon c acid was 130 μM and the incubation medium also contained 3 mM reduced gl tathione and 1 mM EDTA The relat e specif c activity was calculated as (of act ity in the fraction) (of protein in the fract on) Blank values were 0 55 ± S E 0 12 nmol sample All v l es are means ± S E of 4 expts

	Yield in of homogenate	Specific act vity (nmol a achidon c acid con erted m n per mg prote n)	Relati e specific act ity
Homogenate	—	0 098 ± 0 0 7	—
I Plasma membrane fraction	0 31 ± 0 054	0 11 ± 0 017	1 ± 0 11
II Cytomembrane fract on	6 1 ± 0 46	0 1 ± 0 017	4 ± 0 36
III Mitochondr al fract on	5 ± 0 45	0 089 ± 0 014	1 1 ± 0 4
D scard	67 ± 14	0 071 ± 0 018	0 75 ± 0 15
Recovery			

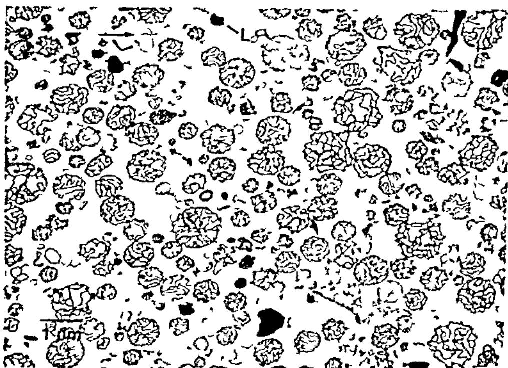


Fig 6 Survey electron micrograph of fraction III (mitochondrial fraction) It consists predominantly of mitochondria which have a dilated intracristal space and a dense matrix Some lysosome like bodies (L) and occasional rough surfaced vesicles (arrow) are also present 10 000

Discussion

Subcellular origin of the membrane fractions

Fraction I was a plasma membrane fraction as judged by the form and thickness of the membranes The low relative specific activities of cytochrome reductase and cytochrome oxidase support this interpretation and indicate that contamination by cytoplasmic membranes or mitochondria was minor

Fraction II contained many of the components often considered typical of the microsomal fraction It has here been referred to as the cytomembrane fraction in order to avoid the often ill defined and misused term microsomes (De Duve 1971) The cytomembrane fraction contained predominantly membranes with a thickness of about 6 nm which often showed a globular substructure (Fig 5) This corresponds to the ultrastructure of the membranes of the endoplasmic reticulum of intact cells (Sjostrand 1963) The high relative specific activity of cytochrome reductase associated with a low activity of cytochrome oxidase (Fig 7) supports the view that the membrane vesicles in the cytomembrane fraction derived from the endoplasmic reticulum (Dallner 1963) and that mitochondrial contamination was low The presence of some 8–9 nm thick membranes indicated that a small amount of plasma membrane fragments were present in this fraction A quantitative estimate of the amount of plasma membranes in the cytomembrane fraction would be of value but as

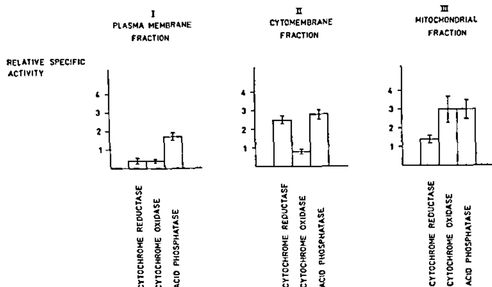


Fig 7 Relative specific activity of marker enzymes in membrane fractions isolated from rabbit renal medulla The relative specific acti ity is calculated as (of activity in the fraction)/(of protein in the fraction) Brackets indicate ± S E.

discussed by De Pierre and Karnovsky (1973) the use of enzyme markers to detect plasma membranes is problematic The most widely used plasma membrane marker is 5 nucleotidase (Benedetti and Emmelot 1968 De Pierre and Karnovsky 1973) but the activity of specific 5 nucleotidase (Ahmed and Reis 1958) was very low in the rabbit renal medulla Furthermore recent observations by Farquhar *et al* (1974) indicate that 5 nucleotid... may also be located in cellular components other than the plasma membrane

The Golgi apparatus is large in some medullary cells (Bohman unpublished) The ...

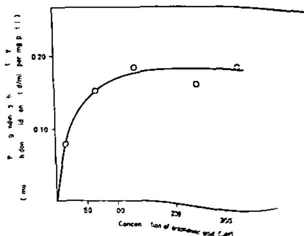


Fig. 8 Effect of different sub strate concentrations on the ac ti ity of prostagland n synthetase in the cytomembrane fract on (II) from the rabb t renal m d lla The cytomembrane fract on (0 40 mg prote n) was incubated at 37 C for 30 min w th d fferent concentratio s of arachidon c ac d

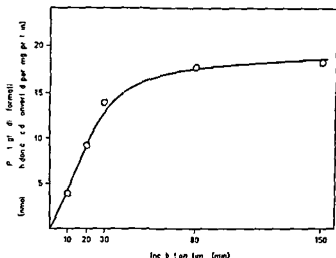


Fig. 9 Time course of prostaglandin synthesis in the cytomembrane fraction (II) from the rabbit renal medulla The cytomembrane fraction (0 41 mg protein) was incubated at 37 C for varying times Substrate concentration was 130 μM

ence of Golgi membranes in the isolated fractions was not monitored by enzyme markers but it is possible as judged from the morphology of Golgi membranes and their density in other centrifugation systems (Reid 1967 De Duve 1971) that part of the cytomembrane fraction was derived from this organelle

Both the ultrastructural and biochemical data show that fraction II also contained some lysosomes

Fraction III was characterized ultrastructurally and biochemically as a mitochondrial fraction containing additionally some lysosomes and a small amount of membrane fragments from the rough endoplasmic reticulum

Procedure for isolation of membrane fractions

Many methods for isolation of plasma membranes are based on the method of Neville (1960) and involve homogenization of the tissue in water or 1 mM sodium bicarbonate

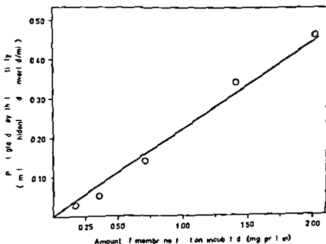


Fig 10 Proportionality of the prostaglandin synthetase assay Varying amounts of the cytomembrane fraction were incubated at 37 C for 25 min with a substrate concentration of 130 μM

However the exposure of the various cell components to hypotonic media may prevent the simultaneous isolation of other subcellular fractions (Reid 1967) In this study therefore a gentle homogenization was carried out in 0 3 M sucrose

The total yield of material in the membrane fractions was low This was mainly due to the extensive and tough framework of basement membranes and other extracellular material present in the renal medulla which was only incompletely fragmented during the homogenization A large part of the cellular material was therefore trapped in this framework and sedimented in the low speed pellet

Assay of prostaglandin synthetase

In order to measure quantitatively the activity of the prostaglandin synthesizing enzyme complex of the rabbit renal medulla we used a radioactive assay with [^{3}H]-labelled precursor Since PGE_2 PGF_2 and PGA_2 are known to be the major prostaglandins of the renal medulla (Daniels *et al* 1967 Lee *et al* 1967) arachidonic acid was used as a precursor (Bergstrom *et al* 1968)

A proportion of the exogenous arachidonic acid was converted to prostaglandin like compounds in the absence of active enzyme in the incubation medium (Nugteren *et al* 1967) The amount of prostaglandin like substances formed non-enzymatically was independent of the incubation time and they appeared to arise mainly during the subsequent extraction and separation procedure The non-enzymatic conversion gave rise to high blank values and thus limited the sensitivity of the method

The somewhat low recovery of prostaglandin synthetase (76%) raises the question whether some physical or chemical inactivation of the enzyme system occurred during the isolation of the membrane fractions However the low recovery may at least in part be attributed to the difficulties involved in the assay of the pooled discard This fraction was very dilute and only about 0 3 mg of protein could be incubated under the conditions employed The determination of such a small amount of enzyme was on the limit of the sensitivity of the method Lands *et al* (1971) reported on a self-catalyzed inactivation of soybean lipoxygenase which may also occur in the initial step of prostaglandin synthesis but it is difficult to judge whether this has had any influence on the present results

Pace Asciak and Wolfe (1970) studying prostaglandin synthesis in rat stomach homogenates found that a considerable dilution of the added substrate by endogenous arachidonic acid took place during incubation and that prostaglandins were formed from both substrates We therefore determined the amount of endogenous arachidonic acid in the different subcellular fractions by gas liquid chromatography (Änggård *et al* 1972) The total amount of arachidonic acid was in the plasma membrane fraction 10 μg in the cytomembrane fraction 43 μg and in the mitochondrial fraction 31 μg per mg protein We have previously shown that the main part was in phospholipids and only about 1/10 in non polar lipids (Änggård *et al* 1972) Since each incubation included about 0 5 mg protein the dilution of the 40 μg exogenous arachidonic acid by endogenous precursor was relatively small Furthermore the dependence of the prostaglandin synthetase activity upon exogenous substrate concentration (Fig 8) did not suggest any major dilution of the added substrate It is believed that at least in some tissues the endogenous esterified fatty acid first has to

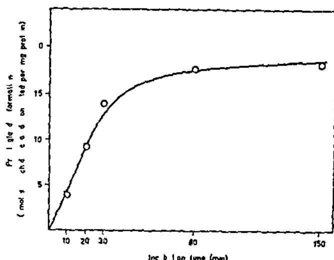


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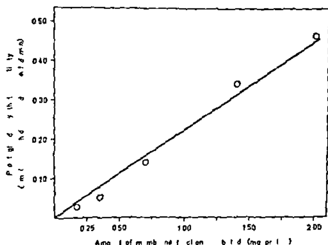


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Acta physiol scand 1975 94 259–270
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Consequences of Myocardial Structural Adaptation on Left Ventricular Compliance and the Frank–Starling Relationship in Spontaneously Hypertensive Rats

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Received 9 January 1975

Abstract

HALLBACK M O ISAKSSON and E NORESSON *Consequences of myocardial structural adaptation on left ventricular compliance and the Frank–Starling relationship in spontaneously hypertensive rats* Acta physiol scand 1975 94 259–270

The Frank Starling relationship of hearts from adult spontaneously hypertensive rats (SHR Okamoto 1969) representing the established phase of hypertension and of young SHR representing the initial phase of hypertension was investigated by using the isolated working heart preparation In the normal diastolic pressure range (5 to 10 cm H O) the left ventricle of both SHR groups displayed significantly reduced stroke volumes compared with hearts of normotensive controls (NCR) the degree of reduction being proportional to the left ventricular hypertrophy This is suggested to be due to a reduced left ventricular diastolic compliance in SHR as indicated by direct measurements of ventricular wall thickness and end-diastolic volumes in arrested hearts exposed to different end-diastolic filling pressures Such a progressive shift of the Frank Starling relationship to the right with duration of hypertension could in combination with the gradual development of "structural autoregulation" of the precapillary resistance vessels constitute dominating factors in shifting the hemodynamic situation in labile hypertension into that characterizing the established or "fixed" state of hypertension

Left ventricular hypertrophy is a generalized finding in established hypertension and its initiation is proposed to be due to the increased tension development per beat when the left ventricle contracts against an enhanced aortic pressure (*cf* Badeer 1964) In the spontaneously hypertensive rat (SHR Okamoto 1969) being one of the best animal analogues for essential hypertension in man left ventricular hypertrophy seems to occur from quite early age in an extent proportional to the increased arterial pressure (Farmer *et al* 1974) Such a structural adaptation to an increased pressure load has also been demonstrated in the resistance vessels in SHR and in patients with essential hypertension (Folkow *et al* 1973 1974) The hemodynamic consequences of such a "structural autoregulation" will be an increased resistance without necessitating any increased degree of smooth muscle shortening due to the presence of an increased wall/lumen ratio in the resistance vessels

In labile hypertension evidently preceding "established" essential hypertension (Eich *et al* 1966 Frohlich *et al* 1969) as well as in the initial phase of SHR hypertension (Pfeller and Frohlich 1973) cardiac output (CO) is increased and total peripheral resistance (TPR) normal (Julius and Schork 1971) The hemodynamic pattern of established essential hyper tension and fixed diastolic SHR hypertension is however characterized by a largely normal CO but a clearly increased TPR though with a tendency of a relative increase in muscle blood supply (*cf* Pickering 1968)

Particularly early phases of essential hypertension often exhibit signs of enhanced adrenergic sympathetic activity (*cf* Julius and Esler 1975) but if this were generalized and uniform it would tend to increase both CO and TPR Since however, neither labile nor established essential hypertension show a hemodynamic pattern of exactly this character some compensatory or autoregulatory adjustments seem to take place or/and the increased sympathetic activity is differentiated in nature The structural adaptation of the resistance vessels large arteries and left ventricle must be considered as perhaps the most important secondary adjustment since it appears to occur so early in hypertension that it becomes of importance both for the initiation and maintenance of the hypertensive state (Folkow *et al* 1973) Concerning the possible involvement of a differentiated sympathetic influence in hypertension the cardiovascular adjustments both in labile hyp rtension and initial SHR hypertension display a similar hemodynamic pattern as that elicited by arousal in normotensive individuals when the defence reaction is initiated Further this reaction inducing a differentiated sympathetic drive on the cardiovascular system is more easily elicited in SHR than in normal control rats (NCR) in a way which reveals an inherent central hyperreactivity to environmental stimuli in SHR (Hallback and Folkow 1974) In the long run such intensified blood pressure increases as initiated by the defence reaction tend to raise the average pressure load on heart and precapillary vessels thereby gradually initiating adaptive structural changes within the high pressure cardiovascular sections characterizing manifest hypertension Thus differentiated and in principle inter mittent rather than tonic and generalized increases in sympathetic activity seem to be involved in the initiation of SHR hypertension

Also adult SHR with manifest hypertension display more vivid defence reactions than age matched controls though the resting steady state is no longer characterized by an increased CO as tends to be the case in earlier phases The present study was therefore undertaken to investigate if an altered Frank–Starling relationship of the hypertrophied l ft heart could at least in part be responsible for the normalization of CO in the established phase of SHR hypertension For this purpose isolated beating hearts from young and adult SHR and matched NCR were perfused at varying end-diastolic filling pressures in an anterograde perfusion system In addition the pressure–volume characteristics of the left ventricle were explored in arrested SHR–NCR hearts.

Methods

Animal Two different age group f mal SHR ere used in the present study Those ref rred to as "young" SHR were 6 week old and weighed approximately 100 g, while those referred to as "adult" SHR were 30 week old and weighed approximately 350 g Age matched NCR were used as controls.

Heart perf s on techn que Before starting the perfusion, arterial pressure was measured in the tail artery during awake conditions (Weiss 1974) During ether anesthesia the hearts were excised and trans ferred to ice-cold salin which stopped the contractions within seconds The aorta was connected to the perfusion apparatus using two different sizes of stainless steel cannulas since the rats belonged to two different weight groups The outer diameter of these thinwalled steel cannulas was such that the aortas had to be widened sl ghtly during mounting the heart. The hearts were then perfused *r a* the aorta with non recirculating Krebs bicarbonate buffer at 37 C (Krebs and Henseleit 193) from a reservoir 70 cm abov the heart, whereupon the hearts immediately started to beat This preperfusion lasted for 5 min during which the left atrium was mounted on another cannula (outer diameter 3 mm) leading to an atrial bubble trap the level of which determined the left ventricular filling pressure (for details see Isaks son 197) Both the aortic and atr al cannulas were connected to Tygon tubes with an inner diameter of 3 mm Following the initial 5 min perfusion period the heart was perfused *via* the left atrium in an anterograde fashion as a working heart preparat on at 37 C (Isaksson 197) To add some distensibility to the rigid aortic tubings an artificial Windkess l in the form of a side-arm f lled with . 5 ml of air was always connected to the tube just above the aortic steel cannula The perfusate entering the left atrium was pumped by the left ventricle to the top of an oxygenating chamber against a hydrostatic pre.sure of 70 cm H O thus allowing the heart to perform a substantial mechanical work *in it o* It is important to point out that with this hydrostatic pressure level above the heart the left ventricular afterload was nevertheless varied to some extent along with alterat ons in cardiac output and thus the cardiac work performance was refl ted both by the output and the consequently modified afterload Cardiac work was changed by varying left atrial f ll ng pressure accompl shed by changing the level between the atrium and the bubbl trap

The rec rculating volume (50 ml in all experiments) was continuously gassed with 95 per cent O_2, and 5 per cent CO Krebs Henseleit bicarbonate buffer was used as perfusion medium in all experiments Final conce trations of the electrolytes in the buffer were (mM) NaCl 118 KCl 4 7 CaCl 2 5 $MgSO_4$ 1 ? KH PO 1 2 and NaHCO 25 The buffer conta ied glucose at a concentration of 14 mM in all e peri ments Coronary flow was measured by collecting the flu d dropping from the heart and ao tic flow was measured by unclamping a s de arm to the aortic tube at the top of the perfus on apparatus close to the oxygenating chamber The pressure head above the heart was recorded *r a* the Windkessel side tube which was connected to a pressure transducer and a Sanborn recorder nodel 16-1300 S After perfusion the left ventricle was cut off placed in a vacuum oven at a temperature of 100 C for days whereafter they were weighed

Determ ation f left t icular compl ance Beat ng hearts of 7 adult SHR and 10 matched NCR anesthetized with ether were excised and dropped into a beaker containing ice-chilled Krebs bicarbonate buffer A cannula with an outer diameter of 4 5 mm was rap dly inserted into the left ventricle *via* the left atrium f tting smoothly into the AV orifi e A narrow rim at the cannula edge made it possible to withd aw the cannula so that its end was placed just at the m tral i sertion after ligation at the AV border Thereafter a bulbous pl sglass rod was placed in the aortic root so that it obstructed the aorta and the coronary orifices without encroaching upon the entricul r cavity thus preventing fluid leakage into the aorta and the coro ary vessels Care was taken to a oid air bubbles entering the left ve tricle during these cannul tion procedures The cannulated heart was then pl ced onto a mo stened ce-cold gauze and the right ve tricle was caut ou ly cut away from the left ventricular preparat on The wide AV can ula was then connected to a stai less steel Y-shaped tube contain g i e-chilled Krebs bicar bonate buffer one end being fixed to a force displacement transducer for conti uous ecording of changes in we ght of the left entricular prep ration while the sidetube was connected *ria* Tygon tubi gs to a reservoir of oxyg nated ice-cold Krebs b arbon te buffer By ra sing or lower ng this reservoir known changes in intra er t icular pressu e could be indu ed as measured via a thin plast c tube inserted ia th AV cannula into the entri le and connected to a Statham press r transduce writ ng on a Grass pol graf To make sure that the l ft entricular preparat ons had not passed to rigor it wa first checked that they responded with contractions to electri al st mulation and when they had regained complete rela at on a standardiz d and rap d pressure volume record ng was performed at const nt temperature cond tion It was repeatedly checked i control preparat ons that the ventr cles w still abl to respond with w ll-coord nated contractions pon el ctri al stimulat o also at the time when th re ord g of the pressure-volume relatio sh ps had been completed F nall to obta n a tru estimat of th ntra e tri cul r diastol ol me at the f lling p essure of 11 cm H O th entr cles were in this tate of ste on immed ately frozen in l q d itrogen

The frozen ventricles were weighed th wn cut open car full blotted and we ghed ag n To simpl fy

the situation the density of the buffer and the left ventri ular wall were considered to be equal and set at 1 The difference between the weight of the frozen ventricle with its buffer content and the weight of the thawn empty ventricle then gives the intra ventricular volume (weight) at the moment of freezing, i e when distended at a pressure of 11 cm H_2O From this value the exact volumes at the different pressures could be obtained from the recorded changes in weight then giving the pressure–volume curve

Statistical analyses were performed according to group comparison t test

Results

Mean arterial pressure was 164 ± 5 mm Hg in the awake adult SHR (n = 13) representing the established phase of hypertension and 135 ± 6 mm Hg in the young SHR (n = 10) representing initial labile phases of hypertension Mean arterial pressures in the corre sponding matched NCR were 119 ± 4 (n = 15) and 110 ± 3 (n = 8) mm Hg respectively (Table I and II) It is also seen from Table I that the ratio between left ventricular dry weight and body weight (mg/g) was approximately 80 per cent greater in adult SHR com pared to age matched controls whereas the corresponding ratio for the young SHR was only some 40 per cent greater compared with their matched controls These measure ments thus demonstrate an increasing degree of left ventricular hypertrophy with extent and duration of hypertension Table I also shows that the intrinsic heart rate which did not change during the course of the experiment tended to be somewhat though not significantly lower in the two SHR groups than in the corresponding NCR

1 *Frank–Starling relationship of adult SHR* Fig 1 illustrates the influence of increasing diastolic filling pressure (DFP) on stroke volume (left part) and on stroke work (*i e* the product of stroke volume and the difference between mean aortic pressure and left diastolic filling pressure right part) of spontaneously beating hearts from adult SHR and matched

TABLE I *Left ventricular dry weight/body weight heart rate stroke work and stroke volume at diastolic filling pressures (DFP) of 8 and 15 cm H_2O of isolated hearts from young SHR adult SHR and matched NCR The ratio between SHR and NCR for each parameter and the degree of significance are illustrated Blood pressure for the respecti e groups are also given* = p 0 05 = p < 0 01 p 0 001 Values presented are means ± S E

	Young SHR (6 weeks)			Old SHR (30 weeks)		
	SHR	NCR	SHR/NCR	SHR	NCR	SHR/NCR
Mean arterial blood pressure mm Hg	135 _ 6	110 ± 3	1 2	164 ± 5	119 ± 4	1 4
Left ventricular dry weight/body weight mg g	0 66 ± 0 0?	0 48 + 0 01	1 4	0 68 ± 0 04	0 37 ± 0 01	1 8
Heart rate beats/min	?81 + 0	308 + 16	0 9	63 ± 0	80 ± 16	0 9
Stroke volume μl						
DFP = 8	82 + 5	94 ± 4	0 9	130 ± 14	17_ ± 10	0 8
DFP = 15	131 + 3	127 ± 6	1 0	249 ± 21	_48 ± 10	1 0
Stroke work g m 10						
DFP = 8	65 ± 5	67 ± 4	1 0	127 ± 15	193 ± 11	0 7
DFP = 15	107 ± 4	86 ± 8	1 3	265 ± 24	_73 ± 4	1 0

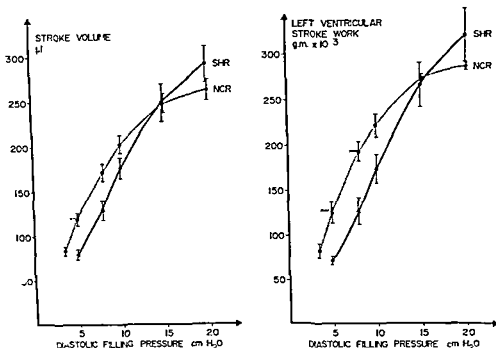


Fig 1 Effect of increased diastolic filling pressure on stroke volume (left part) and stroke work (right part) of SHR aged 30 weeks *i e* "old SHR and age matched controls (NCR) Vertical bars represent ±S E =$p<0\ 05$ =$p<0\ 01$ =$p<0\ 001$

controls A comparison of the right and left parts reveals that changes in stroke work fairly closely reflect changes in stroke volume In the lower range of diastolic filling pressures the SHR hearts produced significantly less stroke work than the NCR ones for a given filling pressure This difference becomes less pronounced at higher diastolic filling pressures since the Starling curve representing the NCR hearts tend to level off at a lower DFP than that of the SHR hearts At the highest DFP tested the stroke work of SHR actually exceeded that of the NCR ones Fig 1 thus demonstrates that in the lower physiological range of diastolic filling pressures a higher filling pressure is required for the SHR left ventricles to produce the same stroke work or stroke volume as the NCR left ventricles

At all levels of DFP the coronary flow/unit dry weight of the SHR hearts was lower than that of the control hearts

II *Diastolic left ventricular compliance of adult SHR* In order to further evaluate the background of the altered Frank–Starling relationship of the SHR left ventricles the compliance of left ventricles taken from 7 adult SHR and 10 age matched NCR were investigated as described in Methods The percentual change in left ventricular volume ($\Delta V/V$) per change in diastolic filling pressure (P) *i e* $\Delta V/V$ ΔP was taken as an index of left ventricular compliance *With the present technique there was no significant difference*

TABLE II Blood pressure left ventricular wet weight, as well as end-diastolic volume and tangential tension per unit tissue mass expressed as relative units $\left(T = \frac{mm\ Hg \times mm}{mm}\right)$ at increasing diastolic filling pressures in adult SHR and corresponding NCR Values presented are means ±S E Levels of significance are given

	Blood pressure mm Hg	Left ventricular wet weight mg	P = 4 5 cm H_2O		P = 7 5 cm H_2O		P = 11 cm H_2O	
			End diastolic volume (µl)	Tension	End diastolic volume (µl)	Tension	End diastolic volume (µl)	Tension
SHR n = 7	164 ± 5	1063 ± 43	249 ± 25	4 0 ± 0 3	353 ± 30	7 8 ± 0 5	377 ± 30	13 1 ± 0 6
NCR n = 10	119 ± 4	782 ± 23	246 ± 10	5 0 ± 0 2	322 ± 14	9 9 ± 0 4	394 ± 16	17 1 ± 0 4
Significance p <	0 001	0 001	0 5	0 01	0 4	0 001	0 3	0 001

between SHR and NCR concerning mean left ventricular volumes at a given end-diastolic pressure over the range of 4 5–11 cm H_2O (see Table I) However the ventricular compliance as calculated for each individual heart tended to be somewhat lower in SHR than in NCR (6 2 ± 0 5 per cent *versus* 7 1 ± 0 5 per cent $p < 0 1$) when the filling pressure was raised from 4 5 cm to 7 5 cm H_2O

The presence of an increased wall thickness in SHR will however in another important way influence left ventricular distention which becomes evident by calculations of average changes in muscle fibre length along the inner mean and outer circumferences of the left ventricles in SHR and NCR These changes in muscle fibre length along the mentioned circumferences were calculated at DFP of 4 5 and 7 5 cm H_2O and are presented as means ±S E in *Fig* 2 The calculations were performed in the following way From the pressure–volume relationship the end diastolic volumes were obtained at the pressures of 4 5 and 7 5 cm H_2O Since the wet weight of the ventricle was determined it was possible to calculate the total ventricular volume at the different pressure levels assuming the ventricle to be a sphere and the density of the buffer and the ventricular wall to be one Since the total ventricular volume i e intraventricular volume plus the volume of the wall was estimated the internal and external radii could be calculated For each ventricle it was then possible to deduce the wall/lumen ratio and also the mean radius corresponding to the midline of the ventricular wall From the values of the inner mean and outer radii the respective circumferences could be calculated Fig 2 demonstrates the changes in left ventricular geometry of SHR hearts (upper circle) and NCR hearts (lower circle) when DFP was increased from 4 5 cm H_2O to 7 5 cm H_2O As a consequence of the increased wall/lumen ratio in the hypertrophied SHR ventricles the percentual increase in average or mean muscle fibre length (l in Fig 2) for this rise of intraventricular pressure is about 20 per cent smaller in SHR than in NCR (p 0 01) When the epicardial muscle fibres are similarly compared those of SHR exhibit only 67 per cent of the distension displayed by

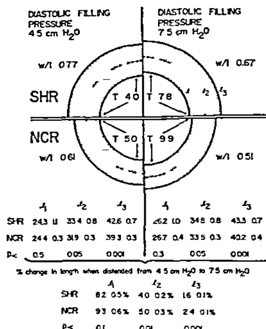


Fig. 2 Theoretical model of a transverse section of the left ventricle from SHR (upper part) and NCR (lower part) The difference between the left and the right part of the figure illustrates the changes in geometry of the respective ventricles when distended from a diastolic filling pressure of 4.5 cm H_2O to 7.5 cm H_2O T represents tangential tension per unit tissue mass ($T = (P \times r)/2 \times w$ = mm Hg) and w/l represents the ratio between wall thickness and lumen *i.e.* internal radius The values $\pm$S E. for the calculated circumferences of the inner radius (l_1) for the "average" or mean radius (l_2) and for the outer radius (l_3) are given for the diastolic filling pressures of 4.5 (left part) and 7.5 cm H O (right part) Below these figures the percentual changes in length of the respective circumferences are given as means $\pm$S E.

the NCR ones (Fig 2) Hence even though the percentual change of the inner radius which by common definition represents ventricular compliance was so slightly reduced in SHR that the difference to NCR was statistically insignificant the reduced elongation of the mean and outer wall circumferences in SHR will influence the Starling relationship as if the SHR ventricular compliance (as defined above) were reduced. In other words most myocardial layers and particularly the epicardial ones will for a given end-diastolic pressure be less distended in the SHR left ventricle than in the NCR one

Furthermore the increased wall/lumen ratio of the SHR left ventricles will also reduce the tangential tension (T) per unit wall thickness at any diastolic filling pressure (P) since according to Frank s modification of the Laplace law $T = (P \times r)/2 \quad w$ where r is the internal radius and w is the wall thickness The mean tangential tension per unit wall thickness thus calculated was significantly lower in the SHR ventricles for any diastolic pressure level ($p < 0.01$–$p < 0.001$) (see also Table II)

III *Frank–Starling relationship in young SHR hearts* In the lower range of diastolic filling pressures SHR display a reduced stroke volume compared to NCR although no difference was obtained at DFP above 10 cm H_2O (Fig 3 left part) However as can be seen from the right part of Fig 3 the left ventricular stroke work of SHR is considerably greater than that of NCR at higher DFP In this experimental setup both resistance and pressure varies with flow so that the afterload is increased at higher CO An increased afterload in SHR for equal flows as in NCR may therefore indicate a greater flow acceleration in the hearts of the young SHR than in NCR.

To summarize Adult SHR with an 80 per cent increase in the ratio of left ventricular

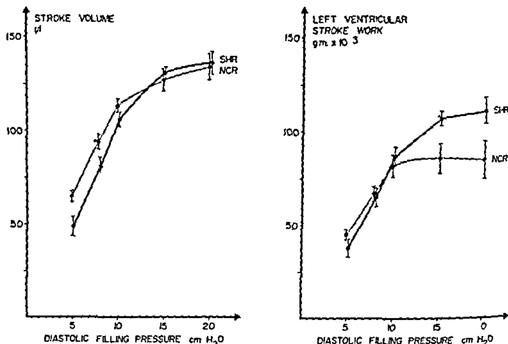


Fig 3 Effe t of increased diastolic filling pressure on stroke volume (left part) and stroke work (right part) of SHR aged 6 weeks *i e* young SHR and age matched controls (NCR) Vertical bars represent ±S E =p<0 05 =p 0 01

dry weight/body weight display a 20–25 per cent reduction in stroke volume compared with NCR at DFP of 8 cm H_2O Young SHR with only a 40 per cent increase in left ventricular dry weight/body weight ratio show only a 10–13 per cent reduction in stroke volume compared with matched NCR Thus particularly in SHR with established hypertension the Frank–Starling relationship of the hypertrophied left ventricles is altered in such a way that a higher end-diastolic filling pressure (or increased inotrophy) is required to produce the same stroke work or stroke volume as in matched NCR *This appears to be due to the increased left ventricular wall thickness in SHR reducing the average diastolic* compliance and tension per unit layer of cardiac muscle and thereby the functional impact of increasing DFP on stroke volume and stroke work In young SHR representing the developing phase of hypertension the mentioned changes are proportionally less pronounced

Discussion

Many studies in man and in SHR have demonstrated a proportionality between the extent of increased blood pressure and left ventricular hypertrophy (*cf* Pickering 1968 Farmer *et al* 1974) In the present study it was illustrated that the left ventricular hypertrophy in SHR has important hemodynamic consequences by displacing the Frank–Starling relation

ship to the right in the lower range of diastolic filling pressure (DFP) the shift being large ly proportional to the degree of hypertrophy Thus in young SHR with some 40 per cent increase of left ventricular mass per body weight stroke volume was approximately 10 per cent lower at equal levels of DFP in the physiological range Adult SHR with an 80 per cent left ventricular increase showed some 20 per cent reduction in stroke volume com pared with NCR at a DFP of 8 cm H_2O (see Table I) The DFP increase required in SHR to produce stroke work or stroke volumes equal to NCR was not a consequence of cardiac dilatation since left ventricular end-diastolic volumes did not differ appreciably between adult SHR and NCR in the physiological DFP range (see Table II) Further the maximal stroke work achieved at the highest DFP investigated was higher for the hypertrophied SHR left ventricles than for the controls particularly in young ani mals

As discussed by Braunwald and Ross (1963) other factors than a depressed myocardial function may well be responsible for an altered Frank–Starling relationship for example a reduced diastolic compliance The present technique for measuring left ventricular compliance (*i e* percentual ventricular volume increase for a given DFP increase) excluded the dynamical impact of the beating heart Defined as above the diastolic compliance of the arrested SHR left ventricle was so slightly reduced compared with NCR ones that the difference was statistically insignificant However the Starling law of the heart implies that the force of contraction is dependent on the *degree of stretch* on the average myocardial fibre (Starling 1918) As suggested by Meerson (1969) equal end-diastolic pressures do not ensure equality of initial fibre length when hypertrophied and non hypertrophied hearts are compared Therefore due to the thicker wall of the SHR left ventricle the end diastolic tension per unit wall thickness will be reduced and the average muscle fibre stretching smaller even when the SHR end-diastolic volume is not significantly smaller than in NCR This is illustrated in the theoretical model of Fig 2 which demonstrates that the percentual elongation of the mean circumference (l in Fig 2) representing the average muscle fibre in the left ventricular wall becomes significantly smaller in SHR than in NCR at equal DFP increases Thus the more the left ventricular wall/lumen ratio is increased by hypertrophy the less pronounced the myocardial prestretching will be for a given DFP increase particularly so in the outer wall layers

When comparing the Frank–Starling curves of young and adult rats (Figs 1 and 3) it can be seen that the myocardium of the young rats reached maximal performance at a lower DFP than that of the adult ones Further the increased stroke work at the higher range of DFP (Fig 3 right part) was in the young SHR not due mainly to an increased stroke volume as in adult SHR but was rather a consequence of an increased afterload Since there was no appreciable difference between young SHR and NCR with respect to cardiac output the higher afterload in SHR suggests a greater flow acceleration when the left ventricles contract *i e* an increased contractility presumably reflecting the greater bulk of myocardium Similar pronounced differences in left ventricular work performance were however not seen in adult SHR and NCR perhaps because the maximal DFP was limited to 20 cm H O at which pressure level the SHR curve was still ascending The left ventricular hypertrophy in adult SHR may however also be admixed with degenerative

myocardial changes *e g* by a higher content of connective tissue (Buccino *et al* 1969 Ooshima *et al* 1972)

Since the tension development rather than the performed work seems to determine cardiac energy consumption (*cf* Meerson 1969) the lower coronary flow in SHR may reflect the smaller tension developed by the SHR left ventricle than the NCR ones at equal DFP Further, the coronary flow of both SHR and NCR increased with increasing cardiac work performance and at higher filling pressures the stroke work of the SHR ventricles exceeded that of the NCR ones despite a lower coronary flow in SHR This indicates that the relatively lower coronary flow in SHR does not determine the reduced stroke work of the SHR left ventricles in the lower range of filling pressures The increased coronary flow resistance of the SHR may also partly reflect a structural autoregulation" of the coronary resistance vessels a process which seems to involve all systemic precapillary vessels in hypertension (Folkow *et al* 1973 1974)

To summarize the above results and considerations the ventricular function curves of the progressively more hypertrophied SHR left ventricles becomes displaced more and more to the right as a result of the increased wall/lumen ratio and reduced ventricular compliance so that they require higher filling pressures in the normal end-diastolic filling pressure range of 5–10 cm H_2O and/or an increased inotropic influence to deliver the same stroke volume as in normotensive controls However the maximal contractile strength of the SHR hypertrophied ventricle is increased as long as no degenerative changes are superimposed

Much interest has been focussed on the role of the sympathetic nervous system in both labile and essential hypertension in man (*cf* Julius and Esler 1975) and in the pathogenesis of spontaneous hypertension in rats (*cf* Okamoto 1972 Folkow 1975) Labile hypertension is characterized by an increased CO but with a seemingly normal TPR (Julius and Schork 1971) whereas in established hypertension CO is normal but TPR increased (Pickering 1968) If the mechanism behind the increased blood pressure in both labile and essential hypertension were only due to a generalized increase of sympathetic tone such differences in hemodynamics would be hard to explain Yet both in labile and essential hypertension heart rate is increased somewhat indicative of an altered neurogenic influence (Frohlich *et al* 1970 Ellis and Julius 1973) According to the present results the impact of an increased sympathetic drive to the heart in hypertension would for a given end diastolic pressure tend to increase or normalize stroke volume depending on the degree of left ventricular hypertrophy which in itself tends to displace the Frank–Starling curve to the right Such a relationship between a decreasing stroke index and increasing left ventricular hypertrophy has been demonstrated also in hypertensive patients without signs of cardiac decompensation (Frohlich *et al* 1971) This is the case also in adult SHR where it is possible to follow more exactly the hemodynamic events during progression of hypertension

It has however been suggested that enhanced adrenergic mechanisms play such an important role in the pathogenesis of SHR hypertension that only neurogenic elements should be involved since the elimination of all sympathetic influences in adult SHR reduces arterial pressure to almost the same levels as in equally treated NCR (Shibayama *et al* 1971) It was however recently shown that in this situation of largely equal low pressures

in SHR and NCR, TPR is still some 35 per cent higher in SHR while CO is correspondingly lower (Albrecht *et al* 1975) In accordance with the present results the structural adaptation of the left SHR ventricle would result in a lower CO in SHR than in NCR at complete cardiovascular denervation a process which would tend to equalize central venous and cardiac filling pressures in SHR and NCR

Human labile hypertension and early SHR hypertension are characterized by a hemodynamic pattern quite similar to that during arousal in normotensive organisms when more or less intense defence reactions are elicited This response pattern which involves neurogenic cardiac acceleration and sympathetic vasoconstriction in all vascular beds except that of the skeletal muscles seems for genetical reasons to be more readily and more powerfully elicited in SHR thereby playing a crucial role for the initiation and progression of SHR hypertension (Hallbäck and Folkow 1974 Hallback 1975) The enhanced sympathetic influence on the SHR cardiovascular system is therefore likely to be in principal intermittent and differentiated in nature rather than tonic and generalized Even if intermittent however an increased sympathetic discharge will raise mean arterial pressure as averaged over days and weeks and such a functional increase of the average pressure load will gradually initiate adaptive structural changes in the left heart systemic arteries and precapillary resistance vessels largely in proportion to the pressure increase The precapillary structural adaptation observed both in SHR and in hypertensive man implies an increased wall/lumen ratio and has the important hemodynamic consequence that increased resistance can be maintained without necessitating any increased excitatory influence (for details see Folkow *et al* 1973 1974)

It is therefore suggested that the hyperkinetic circulatory state of labile hypertension is gradually transferred into that of a normal CO and increased TPR due to the development of the mentioned types of structural cardiovascular adaptation That of the precapillary resistance vessels progressively contributes to the increased TPR while that of the left ventricle gradually shifts the Frank–Starling relationship to the right thereby reducing or dependent on superimposed neurogenic influences normalizing CO

This invest gation was supported by grants from the Swedish Medical Research Council (B74-14X-4250 and B74 14X 16-10C) the Swedish Nation l Association against Heart and Chest Diseases and the Med cal Faculty Uni ers ty of Göt borg The continuous interest and support of Professor Bjorn Folkow throughout this study are gratefully acknowledged AB Hassle generously co ered part of the e penses for a technician

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Acta physiol scand 1975 94 271-277
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Effect of Temperature on Optical Density of Rabbit Blood in Dye Dilution Studies

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Received 20 January 1975

Abstract

ANDERSON N M R K CREASY M DESWIET and K V KAHANPAA *Effect of temperature on optical density of rabbit blood in dye dilution studies* Acta physiol scand 1975 *94* 271-277

The optical density of rabbit blood increased on cooling This increase was additi e to the deflect on produced by indocyanin green at all concentrations measured If calibrat on curves were not corrected for any increase in optical dens ty produced by cooling the blood an error (m nimum 11) was introduced in the estimation of cardiac outpu The effect of temperature was in estigated with three different densito meters Its wave-l ngth dependenc did not follow the absorption spectrum of oxyhemoglobin.

Dye-dilution curves for the estimation of cardiac output in infants and small animals are frequently quantified by performing an on line calibration With th calibration tech nique samples of arterial blood are withdrawn and allowed to stand while different amounts of dye are added to each The samples including a blank sample to which no dye has been added are then reintroduced into a withdrawal line and passed through the cuvette of a densitometer to obtain deflections from a baseline The baseline is established on arterial blood withdrawn directly from the subject

In a series of experiments to determine cardiac output in pregnant rabbits by dye-dilu tion with indocyanine green (Cardio-Green Hynson Westcott and Dunning, Inc Balti more Maryland) we noted that our linear calibration curves in which deflection was plotted on the ordinate showed a y intercept This finding suggested that optical density of blood increased if a delay occurred between withdrawal of the blood and its passage through the cuvette Such a baseline shift if not corrected would introduce an error into the calculation of cardiac output We therefore investigated the cause of the effect in rabbits

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with three different densitometers We found that blood cooled considerably while standing and that cold blood flowing through the densitometers had a greater optical density than warm blood even if no dye had been added

Materials and Methods

Rabbits weighing 3.4 to 5.0 kg were anesthetized i v with sodium pentobarbital (30 mg kg) and were given supplementary xylocaine locally Polyvinyl catheters (internal diameter 0.8 mm external diameter 1.2 mm) were inserted into the femoral arteries at the groin and heparin (250 I U per kg) was administered Blood was withdrawn through the densitometer from the femoral artery with a Harvard pump at 13.5 ml min unless otherwise stated

Three densitometers were used the Waters XP 307 (Waters Company Rochester Minnesota) a dichromatic instrument, and 2 monochromatic instruments the Gilson DTL (Gilson Medical Electronics Middleton Wisconsin) and the Gilford 103 IR (Gilford Instrument Laboratories Oberlin Ohio)

The Waters densitometer was calibrated for use as follows We withdrew a 4 ml sample of blood from the rabbit s femoral artery and either added a measured quantity of indocyanine green or did not add any dye Arterial blood was then withdrawn through the cuvette of the densitometer and a baseline was established on a Beckman direct writing recorder The 4 ml blood sample with or without dye was mixed introduced into the withdrawal line and allowed to pass through the cuvette of the densitometer After a response had been established the blood sample was removed from the system and blood was again withdrawn directly from the rabbit to check the baseline The total time from beginning to obtain the calibration sample until its insertion into the withdrawal line to pass through the cuvette was always less than 2 min. We calibrated the densitometer at 0 2.5 5.0 7.5 and 10.0 mg of dye per liter of blood

In 4 additional studies we performed the calibration eight times at each dye concentration with blood from each of 4 rabbits In 4 of the 8 calibrations the sample was passed through the cuvette within 2 min of its withdrawal (fast) and in the remaining 4 curves the sample was allowed to stand at room temperature for at least 15 min before being passed through the cuvette (slow)

To determine the effect of the temperature of the blood we inserted 2 identical delay loops (polyvinyl internal diameter 0.8 mm external diameter 1.2 mm) between the femoral artery and the densitometer One loop passed through air at room temperature and the other (cold loop) passed through an ice water bath The instrument baseline was established with blood drawn through the loop at room temperature Blood flow was then switched to the cold loop and any change from the baseline was noted The same system was used to study the effect in rabbit plasma. For these experiments the plasma was withdrawn from a beaker in a water bath at 41 C In order to study the effect of temperature at different flow rates the withdrawal speed of the Harvard pump was varied from 5 ml min to 15 ml/min The length immersed of the cold loop was altered so that the degree of cooling was the same at all flow rates

The temperature of flowing blood was measured with a thermistor located in the catheter at the junction of the catheter and the cuvette The thermistor was connected to Wheatstone bridge (Yellow Springs Instrument Company Inc Yellow Springs Ohio) the output of the bridge was displayed on the Beckman recorder The thermistor was calibrated with flowing saline at different temperatures

Results

Dye calibrations with the Waters densitometer were performed on blood from 19 rabbits (15 pregnant 4 nonpregnant) The results confirmed our previous observation of a linear correlation between dye concentration and deflection The mean value of the correlation coefficient was 0.99 For materials that obey Beer s Law optical density is proportional to pigment concentration Therefore over the range of dye concentrations used deflection was proportional to optical density The calibration curves always showed a positive y intercept which could be related to an equivalent dye concentration The 19 intercepts were equivalent to a mean of 1.1 mg/l of dye (S D 0.39) We observed no difference in the

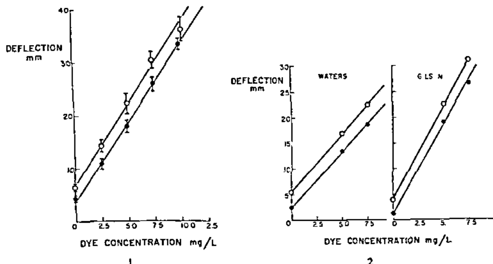


Fig 1 Difference in the slow (○) and "fast" (●) calibrat on curves for no d e and four concentrations of indocyanine green eye obtained with the Waters densitometer on blood from one rabb t (See text) Each point represents the mean ± 1 S D of 4 measurements Regression lines were deri ed by the method of least squares

Fig 2 D fferences in indocyanine green dye cal brations with the Waters and Gilson densitometers with rabbit blood at 0 C (○) and at 2 C (●) Regression l nes were derived by the method of least squares

y intercepts of the curves obtained in the pregnant and nonpregnant animals The magni tude of the y intercept was a function of the time delay between withdrawing the blood samples and passing them through the cuvette of the densitometer In each of the additional four studies in which fast and slow calibration curves were obtained the y intercept of the two (fast and slow) calibration curves differed significantly (P < 0 025 by Stu dent s t test) (See Fig 1) The difference in the means of the intercepts was equivalent to a dye concentration of 0 55 mg/l The slopes of the fast and slow calibration curves did not differ significantly (P > 0 5) indicating that the increment in the optical density of the blood with the time delay was the same at all dye concentrations

Effect of temperature on the optical density of flowing blood

Since the blood samples used for the slow calibration had been withdrawn for a longer time they were cooler than those used for the fast calibration Therefore using the Waters densitometer we investigated the effect of the temperature of blood on its optical density Arterial blood (8 ml) was withdrawn and divided into 2 portions one sample was allowed to reach room temperature (22 C) and the other was immersed in an ice bath Baselines were established as usual The samples were then introduced into the withdrawal line Blood that had reached room temperature showed a deflection representing an increase in optical density equivalent to 1 5 mg/l of dye The sample that had been cooled to 0 C showed a total increase in optical density equivalent to 4 4 mg/l of dye Part of the sample that had been immersed in ice water was then warmed to 41 C (body temperature of the

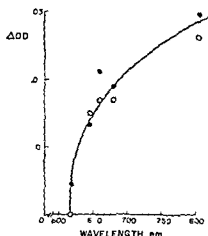


Fig 3 Spectrum of the increase in optical density (O D) that occurred when blood flowed through the cold loop (see text) Symbols represent results obtained on blood from 2 different rabbits

animal) No difference was found between the optical density of the blood drawn directly from the animal and the optical density of the warmed blood

Withdrawal speed

The effect of cooling blood to 22 C was measured with the Gilford densitometer at 805 nm at withdrawal speeds between 5 and 15 ml/min There was no difference in the increase in optical density caused by cooling at different withdrawal speeds

Effect of temperature on calibration curves

Dye calibration curves were obtained with the Waters and Gilson densitometers on blood that had been allowed to reach room temperature (22 C) and on blood standing in ice water Known amounts of dye were added to the samples immediately before they were passed through the densitometer The calibration curves at both blood temperatures for each densitometer showed a shift from the origin to higher optical density (Fig 2) The shift was greater at the lower temperature The curves were shifted in parallel showing that the optical densities due to the cooling effect and to green dye are additive Also the change in baseline due to cooling of the blood did not affect the response of the densitometer to indocyanine green The changes in optical density with cold was in the same direction as the change from fast to slow calibration curves confirming the supposition that the effect of time was an effect of temperature

Wavelength dependence

We studied the wavelength dependence of the optical density change occurring when blood was cooled using the Gilford densitometer and the cold loop described previously The wavelength of the measuring light in the Gilford densitometer was varied by replacing the 805 nm interference filter with 680 660 645 and 617 nm interference filters (half intensity band widths of 16–22 nm) Nonhemolyzed blood cannot be studied at wavelengths less than 617 nm with the Gilford densitometer because the optical density is extremely high

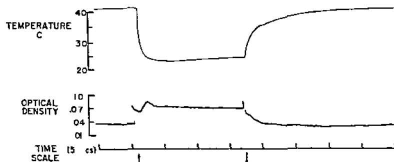
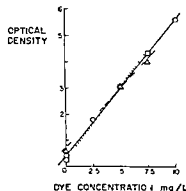


Fig. 4 S multaneous recordings of the changes in temperature and optical density (O D) of blood flow ing through the cold loop (see text) Recordings were made with a thermistor and the Waters densitometer Arrows indicate introduction and withdrawal of cold loop from the system

at the shorter wavelengths The deflection produced by the cold blood was obtained at each wavelength To obtain optical density changes in absolute units at each wavelength we calibrated the densitometer by inserting a neutral density filter with a nominal optical den sity of 0 1 in the light path while blood from the artery was flowing through cuvette The true optical density of the neutral density filter at each wavelength had been established with a Beckman DU spectrophotometer The densitometer deflection obtained with the neutral density filter at each wavelength was used to convert the cold loop deflection to propor tionate optical density units Results obtained on nonhemolyzed blood are shown in Fig 3 In nonhemolyzed blood an increase in optical density was observed at wavelengths greater than 617 nm only

The degree of cooling produced by the cold loop was measured with the thermistor As the temperature of the blood decreased from 41 C to 22 C its optical density increased by 0 03 at 805 nm (Fig 4)

A green dye calibration was also performed at 805 nm with the Gilford densitometer Since the Gilford apparatus was calibrated to yield results in optical density units and since all 3 densitometers had the same sample chamber depth 0 075 cm the optical density value at a dye concentration of 5 mg/l was used to normalize the calibration curves obtained with



F g 5 Comparison of cal bration curves obta ned with 3 den sitometers (Δ) Waters (O) G lford (⁻) G lson The three curves we normal sed to the opt al de s ty 5 mg l of dye shown by the G lford densitometer

the other 2 densitometers The 3 curves are plotted in Fig 5 The y intercept representing the increasing optical density as the blood samples were allowed to reach room temperature was comparable in all 3 densitometers

Discussion

Flowing blood appears to change its optical density at different temperatures Changes in optical density were observed with all 3 densitometers to approximately the same extent and could be eliminated by warming cold blood to body temperature

We considered that changes in the temperature of the sample might introduce chances in the properties of the densitometers For example the dimensions of the sample chamber might change with changes in temperature The sample chambers of the three densitometers however are constructed differently This argues against an increase in path length as the cause of the similar increase in optical density record d by each densitometer Also an in crease in path length suggests that the wavelength dependence should correspond to the absorption spectrum of oxyhemoglobin (Lemberg and Legge 1949) which did not occur (Fig 3) Furthermore there was no difference in optical density of plasma measured between 617 and 805 nm on cooling

We next considered the possibility that changes in the temperature of the sample might alter the response of the detector However in the Gilford densitometer the sample chamber is separated from the photomultiplier tube by 4 5 cm and an optical wedge the temperature of the sample at this distance is unlikely to have any effect on the detector

Blood flowing through narrow tubes shows axial accumulation and changes in red cell orientation (Wever 1954 Taylor 1955 Bayliss 1965 Whitmore 1967) Changes in these 2 factors can be expected with the increase in viscosity that occurs when blood is cooled (Coulter and Pappenheimer 1949) Studies on the light scattering properties of undiluted blood (Anderson and Seklj 1967) have shown that differences in optical density due to light scattering changes should increase as wavelength decreases As shown in Fig 3 the change in optical density did not increase with decreasing wavelength suggesting that changes in red cell orientation did not contribute significantly to the observed changes in optical density It is possible that optical density changes of the kind described by Wever (1954) related to axial accumulation may have contributed to the alterations we ob erved These changes deserve further investigation

Calibration of a densitometer with cold blood samples i not reliable for dye dilution studies unless allowances are made for the effect of temperature All blood samples used for calibration including blood for the baseline determination should be at the same temperature The recording machine is often adjusted so that the optical density of warm blood withdrawn from the animal before the calibration sample is added is taken as zero In these circumstances a deflection will be noted even when the blank sample is run due to the difference in optical density between cold and warm blood This is the source of the y intercept Since the effects of temperature and dye are additive at all dye concentrations the deflection obtained with the blank sample must be subtracted from the deflection obtained at each dye concentration being used in the dye calibration The mean value of the shift

of the baselne due to coolng of blood in our 19 calibration studies was equivalent to 1 1 mg/l of dye If this value had not been subtracted from the calibration curve the error at a dye concentration of 10 mg/l would have been 11° The error would increase with de creasing dye concentration

The error in the calibration curve will falsely decrease the area under the dye dilution curve Therefore in our experiments in rabbits this would have resulted in an overestima tion of cardiac output of at least 11%

The authors wish to acknowledge the advice and suggestions of Dr J I E Hoffman and Dr A M Ru dolph

We are most grateful to Mrs Susan Cowley for typing of the manuscript

This study was supported in part by the United States Public Health Service Grant HE-06285 from The National Heart Institute R K Creasy was supported by Public Health Service Research Training Grant HD-00007 12 M de Swiet and K V Kahanpää were recipients of San Francisco Bay Area Heart Research Committee Fellowship

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Acta physiol scand 1975 94 278-288

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Membrane Potential and Conductance during Pinocytosis Induced in *Amoeba proteus* with Alkali Metal Ions

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Received 22 January 1975

Abstract

JOSEFSSON J O N G HOLMER and S E HANSSON *Membrane potential and conductance during pinocytosis induced in Amoeba proteus with alkali metal ions* Acta physiol scand 1975 94 278–288

An investigation of the relationship between the polarized state of the membrane and the onset and the intensity of pinocytosis was made in *Amoeba proteus* Membrane potential and input resistance was in all instances found to decrease in approximate proportion to the number of channels when pinocytosis was induced by a variety of alkali metal ions at varying pH Channels began to appear when the membrane was depolarized to 30 mV by the inducer of pinocytosis With all inducers the maximum pinocytosis was encountered at membrane potentials close to zero No positive potentials were recorded when the chloride salts of the inducing cations were used At high concentrations of alkali ions a transient increase of the chloride permeability caused short lasting hyperpolarizations of the membrane Inhibition of pinocytosis by Ca was accompanied by an increase of input resistance and membrane potential The selectivity of the membrane to different alkali metal ions observed as changes in pinocytosis intensity membrane potential and input resistance was found to vary with the concentration of the inducer and with the Ca concentration of the extracellular solution Displacement of membrane bound Ca^{+} appeared to decrease the field strength of charged groups in the membrane altering its selectivity among alkali cations The formation of pinocytotic channels is suggested to require translocation of Ca^{++} from the membrane into the cell and would therefore be closely related to the electrical properties of the amoeba

The effects of external Na and K on the transmembrane potential of *Amoeba proteus* has been studied by Bingley and Thompson (1962) and by Josefsson (1966) Increasing the external concentration of either of these ions independently lowered the potential and induced pinocytosis in the cells In both respects Na^{+} was less potent than K^{+} The decrease in membrane potential was accompanied by a reduction in input resistance of the cell

In a recent study (Josefsson 1968) it was observed that monovalent cations induced pinocytosis the order of effectiveness being Cs > K > Na > Li Divalent cations were less active inducers and Ca^{+} and Mn in that order were shown to reduce the sensitivity of the cell to the inducer Ca^{++} and Mn^{+} were themselves without pinocytosis inducing effects

An increase in the sensitivity of the amoeba towards all inducing media was noted when calcium chelating substances were added to the medium or used for the pretreatment of the cells. Inhibition of pinocytosis was also observed when the hydrogen ion concentration in the medium was decreased, although this effect was limited to inducers of low affinity, e.g. to tris, Na^+, Li^+ and to some of the bivalent cations.

Recently Brandt and Freeman (1967) observed that Ca^{++} besides its inhibitory effect on pinocytosis increased the effective membrane resistance of *Amoeba proteus*. Thus there were reasons for regarding Ca^{++} and H^+ to be of significance for the membrane conductance as well as for the hypothetical pinocytosis receptor. These findings prompted us to study the effects of Ca^{++} and pH on membrane potential and input resistance during pinocytosis induced by different inorganic cations.

Methods

Amoeba proteus, fed on *Tetrahymena pyriformis* and starved [illegible] days before an experiment, were cultured in Pringsheim solution at 22 °C according to the technique of Prescott as modified by Chapman-Andresen (1958). The composition of the culture solution and the technique for intracellular recording of membrane potentials were given in an earlier report (Josefsson, 1966).

Electrodes, filled with 3M KCl, were selected for low tip potentials. In some experiments in media of low electrolyte concentration tip potentials were reduced by the addition of 0.05 mM $Th(NO_3)_4$ to the 3M KCl-solution in which the electrodes also were stored.

Input resistance was initially measured using the square pulse technique (Josefsson, 1966). Later, however, a voltage clamp technique was used, which made it possible to measure input resistance at a preset membrane potential. The latter method had technical advantages which made it suitable for the present study. A diagram of the voltage clamp circuit is given in Fig. 1. A single side emitter follower of FET type with high input impedance was used for measuring the membrane potential. The bath was held near ground potential (virtual ground) through a bath electrode connected to the inverting input of the current measuring amplifier. The maximum current to be measured with this device was 15 [illegible] 10^{-4} A. With the electrode used it was possible to successfully apply command signals (V_{cl} in Fig. 1) of ±100 mV except when *the input resistance of the cell was below 0.2 MΩ*. The command signals were taken stepwise from a constant voltage source. The operational amplifiers (Philbrick Inc., USA) required ±15 V power supply.

The input resistance of the amoeba was calculated from the positive or negative current necessary to be injected when the membrane potential was increased by steps of 10 mV from 0 to 100 mV. At low membrane resistances a smaller potential swing was used in order to avoid leakage of the cell membrane. Although the current–voltage relationship did not obey Ohm's law it was considered justified to treat the resistance as ohmic for the purpose of this study.

During an experiment the cells were put in a chamber filled with Pringsheim solution. The electrodes were inserted and membrane resistance was measured by clamping stepwise from 0 to 100 mV within 2 s. Thorough exchange of Pringsheim for test solution was accomplished within 30 s by emptying and filling the bath four times. Approximately 40 ml of the test solution was used for 1 expt. After allowing the cell to reach a steady membrane potential, the transmembrane potential was held at this value by the voltage clamp. Unless stated otherwise all measurements of resistances were made within 5 min after the test solution was applied to the cell. The input resistance of the cells in Pringsheim solution was 9.6 ± 0.75 MΩ (mean ± S.E.) in 53 cells with the voltage clamp technique and 10.5 ± 0.70 MΩ (n = 54) when measured with square pulses. The difference is not significant.

The technique for estimating pinocytosis intensity was the one described by Josefsson (1968). Numerical values for pinocytosis intensity are given as the number of channels developed per amoeba per minute during the first twenty minutes of the pinocytosis cycle. All electrical and morphological studies were made at room temperature ([illegible]) pH was adjusted with HCl or LiOH. The solutions used were made from chemicals of analytical grade. The [illegible] chloride was a Merck Suprapur product. Deionized glass distilled water was used throughout the experiments.

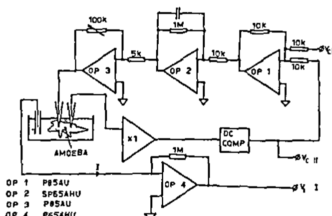


Fig. 1 Voltage clamp circuit diagram The right hand microelectrode was used to record the trans membrane potential The output of the FET emitter follower was connected to a Tektronix 50 oscilloscope and an ink writer (Mingograph EM) and to the inverting input of the first control amplifier (OP 1) With the Amoeba membrane positioned in the feed back loop via the left hand microelectrode the control amplifiers inject positive or negative current to make transmembrane potential match the com mand signal The current was measured with an operational amplifier (OP-4) through an Ag/AgCl—Pringsheim-Agar bath electrode

Results

Correlation between the depolarizing effects and pinocytosis inducing effects of alkali metal cations and NH_4^+

In the present study we found that at pH 5 7 pinocytosis was increased at low concentrations of monovalent cations in the order Rb > K > Cs > Na > Li The dose response curves for these cations are shown in Fig 2 A At higher concentrations the curves for K^+ and Cs^+ intersected to give a new order Rb Cs > K Experiments not included in the figure have shown that pinocytosis induced by these cations in concentrations exceeding 40 mM declines in a manner similar to that shown in the figure for NH_4^+

The effects of the cations on the resting membrane potential of the amoebae are shown in Fig 2 B The sequence of potency was Rb > Cs > K > NH > Na > Li in the concentrations where pinocytosis was being induced Rb^+ being the most potent depolarizer and Li^+ the least active in this respect Pinocytosis (*i e* channel formation) started when the membrane potential reached a value of − 20 to − 30 mV and maximal pinocytosis was observed when the membrane potential was close to zero This occurred at a concentration of 30 mM for Cs^+ and K^+ and at a slightly lower concentration for Rb^+

With increasing concentrations of the cations (above 5–10 mM) the conductance of the cell membrane increased approximately linearly as shown in Fig 3 The conductance increase was greatest with Rb^+ and least with Li The conductance increase followed within seconds of the application of the inducing solution When the conductance was monitored during a full cycle of pinocytosis a *slight increase in permeability occurred with time* but this change could not be related to morphological events

The increase in membrane conductance observed with Cs^+ in 10–20 mM concentrations

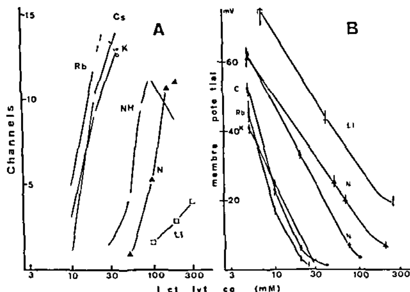


Fig A Pinocytosis versus concentration for univalent cations at pH 5 7 The alues for Rb Cs and K and the values for NH_4 Na and Li were obtained from 2 separate batches of cells

Fig 2B Membrane potential versus concentration of univalent cations at pH 5 7 The measurements for each point were made in at least 15 cells Vertical bars denote ±S E

was less than that induced by similar concentrations of K^+ and Rb^+ (Fig 3) Furthermore in the beginning of the pinocytosis cycle Cs^+ also was less effective than K^+ and Rb^+ in inducing channels at the 10 mM concentration level (Table 1 A) These differences between Cs^+ on one hand and K^+ and Rb^+ on the other could be explained by the effects of Ca^{++} on membrane conductance and pinocytosis Ca^{++} being much more effective in blocking the membrane conductance increase produced by Cs^+ and Na^+ than that caused by the

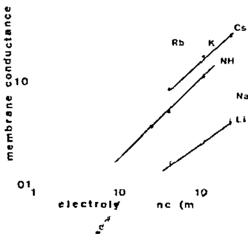


Fig 3 Membrane conductance ersus concentration of uni alent cat ons at pH 5 7 The chlorides of Rb K Cs Na and L were at pH 5 7 wh le NH was at pH 5 5 Where more than fou measurements were a ailable the ±S E a g en as vertical bars

TABLE I A The number of channels observed per amoeba during the first six minutes after application of test solution The number of experiments is given in brackets
B Ca inhibition of the pinocytosis induced by Cs⁺ and K The pinocytosis intensity in solutions containing 0 1 mM $CaCl_2$ is given as percent of the intensity found in corresponding inducing solutions with no calcium added Values are the mean of 2 experiments

		10 mM		20 mM			
A RbCl		14 7 (9)		25 (4)			
KCl		9 7 (9)		27 (4)			
CsCl		1 7 (9)		25 (4)			
B KCl	20 mM	pH 5 0	73	CsCl	20 mM	pH 5 0	11
KCl	0 mM	pH 5 7	62	CsCl	20 mM	pH 5 7	37*
KCl	0 mM	pH 6 5	48	CsCl	20 mM	pH 6 5	23

presence of K⁺ and Rb (Table II) The same applied to the intensity of pinocytosis Ca⁺ was more effective at blocking Cs induced pinocytosis than pinocytosis caused by similar concentrations of K⁺ (Table I B) Inhibition of pinocytosis in the Ca⁺⁺ containing solutions was increased by pretreatment of the cells in Ca ⁺ free solution (0 3 mM NaCl pH 5 7 for 30 min) Considering the great differences between Cs and K⁺ when Ca⁺⁺ is present it appears that pinocytosis induced by these ions could depend on the Ca content of the amoeba membrane at the moment of exchange of solutions

Potassium induced pinocytosis and related membrane potentials

The pinocytosis induced by K⁺ was estimated at four different values of pH (Fig 4 A) and the effect of pH and K ion concentration on the membrane potential was measured in parallel cultures of amoeba (Fig 4 B) In the presence of K concentrations which com[pletel]y depolarized the membrane of the amoeba the greatest number of channels occurred [a]t pH 7 0 5 8 and 4 5 The most intensive pinocytosis and greatest depolarization occurred at pH 4 5 At pH 3 0 very little pinocytosis was observed and the cell was at this pH only partially depolarized irrespective of high potassium concentration

The membrane potentials recorded at K⁺ concentrations above 50 mM were complicated by the observation that the insertion of the microelectrode produced a transient hyperpolarization of the cell of 10–100 seconds duration The hyperpolarizing potential starts

TABLE II Effective membrane resistance measured with voltage-clamp technique in cells immersed in different solutions at pH 5 7 The number of experiments is given in the column marked (n)

	Extracellular solution	(n)	MΩ ± S E
CsCl	25 mM	11	1 8 ± 0 78
CsCl	25 mM 2 5 mM CaCl	15	5 2 ± 0 47
KCl	25 mM	12	1 5 ± 0 21
KCl	25 mM 2 5 mM CaCl	11	2 2 ± 0 76
RbCl	25 mM	4	1 8 ± 0 29
RbCl	25 mM 2 5 mM CaCl	7	2 1 ± 0 16
NaCl	40 mM	4	9 ± 0 74
NaCl	40 mM + 2.5 mM $CaCl_2$	4	9 3 ± 74

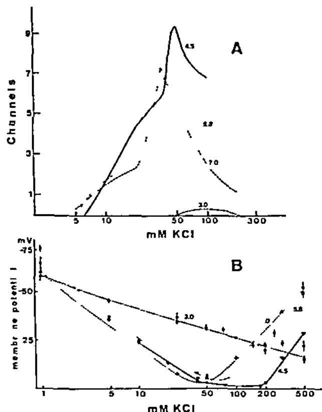


Fig. 4A Intensity of pinocytosis induced by KCl at different pH values indicated in the figures The curves are fitted through the means at the following K concentrations 5 10 0 .5 37 5 40 50 100 and .00 mM (3 to 0 measurements/concentration) The measurements at pH 3 0 are from a single experiment Fig. 4B Membrane potentials in amoeba immersed in 1 to 500 mM KCl at different pH values The mean values for 10-15 amoebae is given with ± S E Measurements from different cultures are the cause of two readings at some concentrations The curves with positive slopes (KCl 50-500 mM) are due to hyperpolarizing potential (see Fig. 5)

immediately but developed slowly and when input resistance measurements were made an increase in membrane conductance was observed Fig 5 A illustrates a typical potential which developed as a result of impaling the cell with a second electrode The input resistance was low until the repolarization began Similar potentials could be produced by intracellular stimulation using depolarizing currents (Fig 5 B) Hyperpolarizing potentials were also observed to occur spontaneously frequently in connection with increased cellular motility

To investigate the cause of the transient hyperpolarization which accompanied the insertion of the microelectrode a study was made in the presence of 300 mM K Th possibility that the potential change was due to chloride current was investigated in media of 300 mM K by increasing the concentrations of Cl and ethanesulphonate in varying proportions In such an experiment the membrane acted as a chloride electrode (Fig 6) The slope of membrane potential against (Cl) was approximately 60 mV per tenfold change of (Cl) concentration at room temperature Pinocytosis intensity was not influenced by these

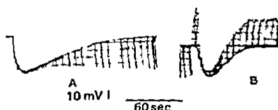


Fig 5 Continuous record of membrane potential on which is superimposed electrotonic potentials resulting from injection of square pulses (current 10 ⁷ A) of 200 msec duration The amoebae were in 300 mM KCl + 2 5 mM CaCl$_2$ Resting potential was zero mV Amplitude of electrotonic potential is proportional to effective input resistance Vertical and horizontal bars indicate 10 mV and 1 min respectively
A Hyperpolarizing potential after insertion of second electrode
B Similar potential induced in this case by outward current from the intracellular stimulating electrode The transient potentials were accompanied by a decrease of input resistance

potentials Hyperpolarizing potentials also appeared in Ca^{++} free K^+ solutions but were of higher amplitude and of longer duration in the presence of Ca^{++} In this respect Sr^{++} could not substitute for Ca^{++} (Fig 6) The presence of group 1 A metal cations favoured the development of the potential (Table III) The order of efficacy in this respect seemed to be that of free solution mobilities (Cs > Rb > K > NH_4 > Na > Li) Pretreatment of the amoeba with a solution containing a calcium chelator inhibited the potential as well as the rush of cytoplasm towards the electrode which usually followed the impalement

Addition of Ca^{+} to the external medium of *Amoeba proteus* inhibits induced pinocytosis in a competitive manner (Cooper 1968 Josefsson 1968) and increases the effective resistance of the cell (Brandt and Freeman 1967) To investigate concomitant changes in the membrane potential Ca^{++} was added to a solution containing NaCl (100 mM at pH 7 0 or 200 mM pH 5 8) or KCl (40 mM at pH 5 8 or 7 0) These pH s and ionic concentrations were chosen because they induced a high level of pinocytosis and were within the ascending part of the dose response curves for respective ion In these media the addition of Ca caused an increase in the membrane potential of the cells as shown in Fig 7 A The time course of the

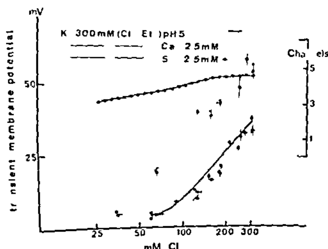


Fig 6 Transient membrane potentials recorded in 300 mM K salt solutions at pH 5 0 The chloride concentration (abscissa) was aried by substitution of chloride for ethanesulphonate The addition of Ca + made the potentials appear at a lower chloride concentration Extrapolation of this curve indicated an intracellular chloride concentration of approximately 25 mM The broken line in the middle of the graph shows the level of pinocytosis intensity in the 300 K 5 Ca solution The slightly weaker pinocytosis at low chloride concentration is probably due to the Ca binding activity of the ethanesulphonate ions

TABLE III Transient potentials (mV) as measured in 500 mM of the chlorides of different alkali ions at pH 5 7 The means ±S E are given The number of experiments is within the brackets

Cs	57 4±2 13 (17)	NH_4	27 7±1 42 (38)
Rb	56 2±3 58 (19)	Na	17 4±0 79 (33)
K	44 4±1 81 (24)	Li	16 2±1 03 (33)

repolarization was similar for the Na^+ and K^+ solutions when the experiments were performed at pH 5 8 At neutral pH however the effect of Ca^{++} was more pronounced in 100 mM NaCl than in 40 mM KCl As shown by Fig 7 B increasing concentrations of Ca^{++} gradually inhibited pinocytosis induced in the presence of KCl or NaCl solutions With the exception of pinocytosis induced by K^+ at pH 7 0 1 mM Ca^{++} completely inhibited pinocytosis The inhibition seemed to follow a linear course in the semi log plot A similar relationship was found for membrane potential versus increasing Ca^{++} concentrations In the experiments with the addition of Ca^{++} at pH 5 7 inhibition of pinocytosis occurred at a level of membrane potential lower than that necessary to induce pinocytosis 30 mV compare Figs 7 A and 7 B with 2 A and 2 B

Discussion

The data reported on the potentials measured in *Amoeba proteus* are in accordance with the picture that the amoeba membrane is permselective to cations (Josefsson 1966) The membrane potential was dependent on the concentration and species of the monovalent cation in the extracellular solution and on the pH of the solution Thus a tenfold increase of K concentration at pH 5 7 caused a depolarization of 50 mV This level of depolarization which is larger than that earlier reported (Josefsson 1966) probably reflects a different technique for impaling cells As a comparison the values for resting potentials in Pringsheim was around −80 mV instead of the −65 mV reported earlier

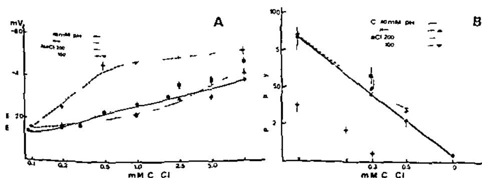


Fig. 7A Membra e potent al (ord nate) measured in KCl 40 mM (pH 5 8 a d pH 7 0) N Cl 100 mM (pH 7 0) and 00 mM (pH 5 8) To these solutio C Cl was added to gi e incre s ng concent at ons up to 10 mM (abscissa) V rti al bars denote ±S E

F g. 7B Inh b tion of pinocyto s by Ca Pinocytos nt ns ty is gi en as percent of channels obser ed in respective induc ng solut on w th no Ca added Each po t is a rage of 4–11 e per ments the vertical bars indicating two t mes the sta dard error of the means.

The slope for depolarization against increasing concentrations of K^+ (pH 5.7) approached that predicted by the Nernst formula (i.e. the membrane is a K^+ electrode). At high hydrogen ion concentrations (pH 3.0) the slope was markedly decreased which may signify that the membrane is becoming permeable to anions.

During the transient hyperpolarizing response to mechanical stimulation the amoeba behaved like a chloride electrode for [Cl⁻] < 25 mM and the amplitude of the hyperpolarizing potential increased in the sequence determined by the ionic crystal radius of the cation present. Consequently the original exclusion of the Cl anions from passing through the membrane decreased as the cation radius increased (i.e. Cs > Rb > K > Na > Li). Simultaneously with the change in potential a decrease in transmembrane resistance was observed. The presence of Ca^{++} but not Sr^{++} increased the sensitivity of membrane potential to Cl. This might reflect the stabilizing effects of Ca^{++} (not demonstrated by Sr^{++}) which may involve a "sealing" of the membrane around the microelectrode tip after penetration and thus a decreased permeability to cations. The potentials observed by Lassen *et al.* (1974) after puncture of *Amphiuma* red cells were increased by Ca^{++} which was suggested to have an intracellular effect. Similarly Ca^{++} diffusing into the amoeba may increase the Cl permeability of the membrane and subsequent sequestering of Ca^{++} may explain the transient nature of the potential.

The study of the input resistance as measured with the voltage clamp technique gave no evidence for abrupt increase of conductance when the concentration of the external electrolyte was increased to the level where pinocytotic channels develop. There was an approximate quantitative relationship between the number of channels induced and the value of the input resistance. Channel formation commenced at an input resistance of 4 MΩ and intensive pinocytosis (i.e. > 10 channels per amoeba and minute) was observed when it was approx. 1.5 MΩ.

The order in which cation species induce pinocytosis and the membrane permeability to these cations could in the light of recent theories presented by Eisenmann (1962, 1965) give information on the nature of negative membrane sites. As shown by Eisenmann (1963) the sequence of selectivity for cation species in several biological systems appears to follow the "field strength theory" of ionic specificity of glass electrodes (*cf.* Diamond and Wright 1969). The permutations of sequence for Cs, Rb and K obtained in the studies of pinocytosis (Fig. 2 A), membrane potential (Fig. 2 B) and transmembrane conductance (Fig. 3) were in accordance with some of those suggested by Eisenmann. As the cation concentration was increased these three parameters exhibited a gradual transition of sequence permutation, IV (K > Rb > Cs) → III (Rb > K > Cs) → II (Rb > Cs > K) indicating a decreased anionic field strength of the cation detecting structures present in the membrane. Sequence I (Cs > Rb > K) observed by Josefsson (1966) was never attained in this study. The curve for conductivity passed through the first (IV → III) and pinocytosis passed through the second (III → II) of these transitions while the selectivity order for membrane depolarization included both (IV → III → II). In the membrane potential measurements the transition in sequence permutation occurred at lower concentrations than in the conductance studies. This was probably due to the difference in duration of the two types of experiments and might indicate an effect of Ca^{++} on the ionic selectivity of the membrane. The Ca^{++} concentration of the

membrane is likely to decrease with time in the medium Likewise a decrease of the membrane bound Ca^{++} can be expected when the external concentration of alkali cations is increased A conformational change in the membrane of the amoeba during induction of pinocytosis is suggested from two recent observations Swelling of the amoeba membrane was observed by Brandt and Freeman (1967) when the input resistance was low and the pinocytosis intensive and a general increase of permeability was found to be produced by cations in proportion to their efficacy as inducers (Brandt and Hendil 1970 1972) The altered membrane selectivity which occurred when the concentration of cations was raised might indicate that structural changes are caused by univalent-divalent ion exchange in the membrane Release *of* Ca^{++} *from the membrane into the cytoplasm could be the mechanism which* relates permeability and pinocytosis to the conformational changes

In general the correlation between intensity of induced pinocytosis and membrane polarization was good Inhibition of pinocytosis by changes of Ca^{++} concentration in the medium or by altering the pH of the external solution were in all instances accompanied by an increase of membrane potential The channels appeared when the potentials were 30 mV or less but the addition of Ca^{++} seemed to inhibit pinocytosis before the membrane potential was brought back to this level Moreover at a given concentration of alkali metal cations the permutation sequence number was higher for pinocytosis than for the membrane potential This could mean that exchange of ions occurs at sites relevant for pinocytosis but different from those which determine the permeability characteristics of the membrane A simultaneous comparison between the bioelectrical parameters and the pinocytosis intensity measured on the same cell would settle this question It has been claimed from the study of pH optima of the cation induced pinocyto is (Josefsson 1968) that certain membrane groups may constitute receptors for certain cation inducers of pinocytosis Thu tris and Na^{+} will induce pinocytosis at pH 7 while K^{+} is most effective at pH 5 6 Without postulating specific receptors for simple cations this finding may be explained in terms of the field strength theory by the difference in sequence permutation among cations in membranes of high (at pH 7) and low anionic field strengths

Depolarization of the amoeba membrane probably starts a chain of events about which we have little knowledge leading to invagination of the cell membrane Ca^{+} is required for channel formation and regulates the membrane potential and input resistance of the cell displacement of Ca from the membrane causing a change in these three parameters Translocation of Ca from the cell membrane into the cytoplasm may be an intermediate step in the chain of events between addition of the inducing cation and channel formation This mechanism may explain why the pinocytosis induced by alkali metal cations is reflected by the electrical properties of the cell

This work was s pported by g ants f om the Medical Faculty Uni ers ty of Lund and gra t no 167 K68-01X from the Swedish Cancer Soci ty

References

BINGLEY M S and C M THOMPSON B oelectric potentials in relation to movement in amoeba *J Theo B l* 1962 2 16–3

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Acta physiol. scand 1975 94 289-300
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The Pressure-Flow Relationship of Different Nephron Populations in the Rat

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Received 25 October 1974

Abstract

KALLSKOG Ö L O LINDBOM H R ULFENDAHL and M WOLGAST *The pressure flow relationship of different nephron populations in the rat* Acta physiol scand 1975 *94* 289-300

The mechanisms behind the autoregulation of the total renal blood flow and the glomerular filtration rate are unclear In this investigation a modified microsphere technique was applied to measure the blood flow at different depths in the renal cortex during normotensive and hypotensive conditions No autoregulation was found in the outer cortex while it was well pronounced in the inner one During similar conditions glomerular capillary pressure welling point pressure and intratubular pressure were recorded By combining these results with the blood flow data the preglomerular and postglomerular resistances were calculated It was then found that the preglomerular resistance decreased and the postglomerular resistance increased when the blood pressure was lowered The results indicate a redistribution of blood flow from the outer parts to the inner parts of the cortex when the blood pressure is decreased The redistribution of the blood flow might explain the well known linear relationship between the arterial pressure and the urine flow The single nephron filtration rate of the outermost glomeruli could be calculated and the results seem to indicate a non-equilibrium at the end of the glomerular capillaries

The pressure-blood flow relationship in the kidney is characterized by an essentially unchanged blood flow when the perfusion pressure in the renal artery varies between some 70 and 200 mm Hg (Thurau 1964 Waugh 1964 Hinshaw 1964 Loyning 1971) This phenomenon has been referred to as the autoregulation of the renal blood flow Also the glomerular filtration rate (GFR) is autoregulated within approximately the same pressure range (Forster and Maes 1947 Selkurt *et al* 1949 Thompson *et al* 1957) It has been concluded that the mechanism behind the autoregulative response is localized in the afferent arterioles

The finding of an autoregulation of the total renal blood flow does not obviously rule out the possibility that some of the nephron populations do not exhibit this response

In the present study the blood flow in different nephron populations during normo- and hypotensive conditions was investigated by means of a modified microsphere technique with microdissection of single glomeruli The renal perfusion pressure was changed by an aortic clamp between the two renal arteries The results indicate that the deeper the glomeruli the more effective the autoregulation of blood flow The superficial glomerular blood flow was nearly linearly related to the perfusion pressure This might then mean that a de

creased perfusion pressure leads to a redistribution of the blood flow from the superficial salt loosing glomeruli to the deep salt saving ones (Goodyear and Jaeger 1955 Barger 1973) This phenomenon might at least partly explain the well known linear relation between urine and sodium excretion and the perfusion pressure (Selkurt 1951)

In an additional study measurements of the glomerular capillary pressures and the welling point pressures were made By combining these results with those of the blood flow measurements in the superficial glomeruli the pre and postglomerular resistances were calculated During decreasing blood pressures the afferent arteriolar resistance was found to decrease while the efferent arteriolar resistance increased

Material and Methods

All experiments were carried out on male Sprauge Dawley rats weighing between 265 and 3 0 g The rats were anaesthetized with intraperitoneal administration of Inactin® (Chemical Fabrik Promonta GmbH Hamburg, West Germany) in a dose of 120 mg/kg b wt tracheostomized and placed on a servo-controlled heating pad The left kidney was exposed by a flank incision and placed in a lucite cup The kidney was covered with a rice paper drenched with mineral oil A clamp was placed on the aorta between the renal arteries In the microsphere experiments a catheter was inserted into the right carotid artery and placed with its tip at the aortic root just above the aortic valves Blood was drawn from a catheter in a femoral artery with a suction pump (Syringe pump Model 351 Sage Instrument USA) at a rate of 0 6 ml min while the arterial pressure was monitored in the other femoral artery In some experiments the pressure was measured directly in the renal artery via a 200 μm steel cannula (Källskog *et al* 1975) This pressure was only slightly below the one which was recorded in the femoral artery

The spheres labelled with 141 Ce and 85 Sr (3 M Co Saint Paul Minn USA) were separated by a sedimentation procedure in large glass cyl nders as described previously (Källskog *et al* 197) by which the ize scatter could be reduced to about half of the original value (15±5 μm, mean±S D) About 0 5 mill ieres of either kind (as a suspension in 0 5 ml of plasma) were injected the injection time was about 20 s) The suction pump was started just before the injection and was stopped about 15 s after the injection of the sphere suspension

The first microsphere injection was performed during control normotensive conditions as a rule with the use of the 141-Ce labelled batch The perfusion pressure was then reduced to the desired level followed by the injection of the 85 Sr labelled spheres After completion of the experiment the abdominal aorta was catheterized with a relatively large catheter through which liquid silicon rubber (Microfil Canton Biomed Prod Inc Box 2017 Boulder Colorado 80307 USA) was injected in order to visualize the renal vascular tree

The kidneys were excised and macerated at 40 C for 30 min in 50 hydrochloric acid After rinsing the kidneys were embedded in carboxymethyl cellulose frozen to −20 C and in a microtome cut into 400 μm thick sections From these sections 50 or more superficial and true juxtamedullary glomeruli respectively were sampled (Bankir 1973) and analyzed with respect to their 141-Ce and 85 Sr activities in a gamma spectrometer (Nucab AB P O 2309 S-421 02 Västra Frölunda Sweden) The true juxtamedullary glomeruli were defined as those giving rise to vasa recta. The sections were then cut perpendicularly to the striatal direction in four cortical zones as according to Fig 1 The glomeruli in the deepest part are both true juxtamedullary ones but also includes those which do not gi e rise to vasa recta

The blood flow in the samples was calculated by multiplying the sampling rate of 0 6 ml min with the sample activity over the activity in the constantly withdrawn reference blood sample For card ac outpu determinations the sample activity was replaced by the total amount of the activity injected Th s blood flow value then refers to card ac output minus the coronary blood flow The total blood flow in the region for which a zone is representati e could then be calculated by multiplying the total renal blood flow with the quotient between the acti ity of this zone o er the summated acti ity of the four zones

Micropunctu e procedure

In further experiments the pressure characteristics in the cortical vascular tree and in the proximal tubules were investigated The pressures were determined with a servo-controlled counterpressure measuring

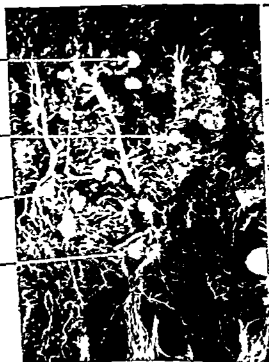


Fig. 1 The picture shows a microphotograph of the cortical part of the rat kidney The vascular bed is filled with silicone rubber and the 100 μm thick section was previously immersed into solutions with an increasing concentration of ethanol and then in methylene salicylate The different types of glomeruli I II III a and III b are noted to the left. The brackets to the right show the different zones cut from the sections

device (according to Wiederhielm (1964) as modified by Intaglietta (1970)) connected to a sharpened glass capillary with a tip diameter of about 4 μm Glomerular capillary pressure was measured indirectly by the stop-flow method according to Gertz (1966) For this purpose an early proximal tubule was punctured with a relatively thick cannula through which mineral oil was injected until the glomerular filtration ceased The stop flow pressure thus induced was measured with a second pressure cannula inserted proximally to the oil column From this pressure the glomerular capillary pressure was obtained by adding an oncotic pressure of 22 mm Hg corresponding to a systemic plasma concentration of 6 per cent (Landis and Pappenheimer 1963) The lowest possible perfusion pressure investigated was about 50 mm Hg under which pressure the filtration vanished and the tubules were collapsed.

Results

Blood flow data

All blood flow data are brought together in Table I and are summarized in Fig 2 3 and 4

Fig. 2 shows the relation between the perfusion pressure and the total renal and regional blood flow in the left experimental kidney Experiments with weak or no autoregulation with respect to the total renal blood flow are excluded (marked with an asterisk in the table)

A further treatment of these excluded experiments showed that this weak autoregulation was valid even for the regional blood flow and in addition was mainly not accompanied by a redistribution of the blood flow The lines drawn represent the [illegible] the parabolic

TABLE I Perfusion pressure cardiac output total and regional renal blood flows during control normotensive conditions and during the reduction of the perfusion pressure to the left experimental kidney Experiments denoted with an asterisk represent non autoregulating kidneys with respect to the total renal blood flow

Rat weight g	Perfusion pressure mm Hg		Cardiac output ml/min 100 g BW		RBF exp kidney ml/min 100 g BW		Blood flow in cortical zones experimental kidney µl/zone								Superficial glomerular flow nl/min 100 g BW		Juxtamedullary glomerular flow nl/min 100 g BW		RBF control kidney ml/min 100 g BW	
							1		2		3		4							
	pre clamp	clamp	pre clamp	clamp	pre clamp	clamp	pre clamp	clamp	pre clamp	clamp	pre clamp	clamp	pre clamp	clamp	pre clamp	clamp	pre clamp	clamp	pre clamp	clamp
318	118	78	24 3	21 9	1 83	1 38	206 2	83 3	104 5	68 1	106 5	100 4	76 9	75 9			76 2	53 1	1 89	1 60
309	100	74	27 1	25 4	2 20	1 91	151 2	95 3	100 5	92 5	73 8	94 7	72 0	90 6			84 5	63 4	1 71	1 39
288	117	54	32 6	21 4	1 60	0 90	136 2	54 6	66 7	43 9	40 6	45 5	39 0	44 1			74 3	74 0	2 71	1 98
271	102	44	34 6	27 7	2 36	0 96	197 6	66 5	139 7	51 9	91 9	55 6	81 1	51 4			93 7	77 1	2 40	1 72
290	117	31	29 0	18 7	2 86	0 72	317 4	28 0	239 3	33 2	174 2	36 8	104 6	25 7			156 7	32 1	1 66	1 62
290	107	56	34 9	35 7	2 38	1 72	243 2	123 0	115 6	99 0	88 5	99 7	59 1	80 8			58 3	115 6	2 90	2.03
285	120	17	46 3	12 5	3 02	0 14	393 9	22 4	192.9	10 4	139 8	10 1	113 0	10 1			78 6	12 3	1 86	0 95
270	124	78	37 8	28 2	2 03	1 71	184 1	106 0	106 7	98 3	80 2	94 4	53 7	63 5	135 6	83 0	57 0	78 9	2 29	1 54
263	102	19	31 3	17 4	3 52	0 24	215 1	6 3	145 5	10 7	77 0	8 3	94 9	8 0	122 1	4 9	129 3	7 2		
263	102	19	31 3	17 4	2 11	0 20	222 5	11 2	162 4	5 6	93 7	7 3	69 5	5 1						
265*	113	81	33 0	32 5	2 65	1 99	190 8	80 0	106 8	89 4	86 5	77 2	85 1	81 0	105 3	48 3	64 5	74 3	2 57	1 90
307	105	21	20 6	22 7	2 40	0 22	94 8	11 2	90 0	16 7	75 2	7 1	90 6	14 2	97 1	21 2	146 3	13 4	1 92	1 39
285	110	21	42 8	19 5	3 13	0 28	211 7	14 6	121 6	14 3	98 2	12 0	93 0	15 3	202 8	17 9	183 2	21 4		
285	110	21	42 8	19 5	2 79	0 17	275 6	5 0	102 1	3 9	64 9	5 8	50 2	3 2						
298	125	82	42 2	30 2	3 63	2 86	311 9	134 1	107 9	103 1	59 1	87 8	60 5	65 4	325 5	150 3	167 8	189 3	1 51	1 34
302	125	80	30 6	19 2	1 68	1 12	82 0	33 9	48 8	37 7	32 2	31 8	23 2	20 4	110 9	55 3	80 8	81 4	1 63	0 85
313	127	76	23 9	0 9	1 38	1 28	46 3	33 0	52 2	39 7	41 2	43 4	33 8	31 1	93 9	56 9	80 5	71 9	1 79	1 44
306*	105	75	27 3	21 0	2 53	1 83	94 9	56 7	87 7	60 0	46 6	49 2	68 5	71 2	178 8	107 8	152 6	87 9	1 43	1 65
286	130	80	33 5	27 8	1 90	1 31	78 3	52 3	52 4	58 5	34 0	38 3	26 9	24 6	115 7	73 8	105 9	81 1	0 75	0 04
269*	116	91	33 5	30 5	2 15	1 65	160 5	57 8	110 0	105 7	54 0	76 4	32 9	41 4	137 9	84 0	130 5	97 0		
289	132	57	30 2	23 0	1 69	1 8	43 7	22 2	36 3	27 4	23 0	23 7	32 5	30 2	83 4	65 1	97 6	100 0	2 37	1 84
271	127	50	29 9	27 1	1 54	0 89	53 4	17 5	34 6	23 0	19 6	18 3	27 1	19 4	115 1	43 2	61 3	62 7	1 93	1 68
68	127	78	27 5	19 2	2 81	2 25	177 4	87 4	62 2	77 3	38 2	68 7	43 4	55 9	155 2	98 5	59 3	83 2	2 87	2 07
273*	112	60	50 5	40 7	2 48	1 18	140 2	29 8	86 6	25 7	56 6	32 2	62 4	40 0	173 3	26 4	124 5	48 4	4 18	2 92
89	117	83	24 8	27 8	2 05	1 67	117 8	73 7	92 3	99 3	40 2	58 1	59 6	97 1	140 5	85 8	94 1	139 1	1 78	1 42

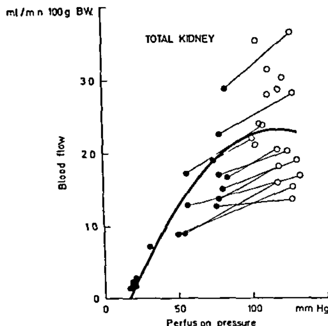


Fig. 2 a. Autoregulation of blood flow in the total kidney The open circles represent the pre-clamped values and the filled circles the clamped values

relation $y = a_0 + a x + a_2 x$ as calculated with the least square method The blood flow–pressure relationship of the superficial cortical zone seems not to deviate from a linear relation while the curves of zones 2 3 and 4 show an autoregulatory pattern In the inner zones there is a tendency to an increased blood flow at a moderate reduction in the perfusion pressure Utilizing all data from zones 3 and 4 this increase was shown to be significant ($0.01 < P < 0.02$) The values of the single glomerular blood perfusion rates show quite the same picture as the corresponding zones In Fig 3 the fractional blood flow in the cortical zones are related to the arterial blood pressure and it is seen that a reduction in the arterial blood pressure from the control values to about 70 mm Hg resulted in a redistribution of the blood flow A relative decrease is clearly seen in zone 1 and concomitant increases are observed in zones 3 and 4 while zone 2 was unchanged This distribution remained essentially unchanged by a further reduction in the perfusion pressure

In Fig 4 the percentage change in cardiac output total and regional blood flow in the right control kiney are depicted The figure shows that the experimental manoeuvre in itself will be concomitant with a moderate reduction of these blood flows in parallel with a reduction in the cardiac output

Micropuncture data

The hydrostatic pressures within the superficial cortical vasculature and the proximal tubules are depicted in Table II The pressures in the glomerular capillaries and the welling points are shown in Fig 5 From this figure it would seem that both the glomerular capillary pressure and the welling point pressure are autoregulated A similar relation is also found for the peritubular capillary pressures as well as the proximal tubular pressures For

TABLE II Hydrostatic pressures within the superficial cortical vasculature and the proximal tubules of different arterial perfusion pressures

Rat Weight g	Condition	Perfusion pressure mm Hg	Glom capl pressure mm Hg	Welling point pressure mm Hg	Peritub capl pressure mm Hg	Tubular pressure mm Hg	Init filtrat. pressure mm Hg	Pressure drop aff arteriole mm Hg	Pressure drop eff arteriole mm Hg
1	control	118	68 0	13 0	12 0	15 0	31 0	50	55
286	clamp	81	53 0	13 0	9 0	13 3	17 7	28	40
	control	132	61 0	15 0	—	14 6	24 4	71	46
	clamp	108	59 0	14 0	10 0	12 8	24 2	49	45
	clamp*	73	48 0	—	—	11 8	14 2	25	—
2	control	114	56 6	10 5	—	11 5	23 1	57 4	46 1
298	clamp	72	46 8	12 3	11 5	11 8	13 0	25 2	34 5
	control	127	64 0	18 0	—	19 3	22 7	63 0	46 0
3	control	118	55 5	13 3	9 3	11 5	22 0	62 5	42 2
280	clamp	81	48 1	12.9	8 5	11 8	14 3	32 9	35 2
	control	106	54 2	12 8	9 5	12 1	20 1	51 8	41 4
	clamp	65	40 5	13 5	9 3	11 3	7 2	24 5	27 0
4	control	118	53 9	14 1	9 5	10 1	21 8	64 1	39 8
290	clamp	79	42 1	12.5	6 5	9 4	10 7	36 9	29 6
	clamp	69	40 5	8 0	—	9 8	8 7	28 5	3? 5
	clamp	55	31 0	10 0	—	6 0	3 0	24 0	21 0
5	control	115	52 0	15 0	11 3	9 4	20 6	63 0	37 0
310	clamp	76	42 0	13 5	7 5	9 8	10 2	34 0	28 5
	control	100	55 2	—	10 0	10 5	2 8	—	—
	clamp	56	38 9	12 2	9 5	10 2	6 7	17 1	26 7
6	control	118	57 7	13 5	9 5	12 3	21 4	60 3	44 2
260	clamp	76	50 7	11 5	7 3	10 7	18 3	25 3	39 2
	clamp	26	—	4	—	—	—	—	—
	control	118±7 5	58±5 3	14±2 1	10±1 2	13±3 0	23±3 3	60±6 5	44±5 1
±S D	clamp	74±14 4	45±7 7	12±1 8	9±1 5	11±2 0	12±6 1	30±8 5	33±7 0

rat kidney and the results are contradictory Thus Ohler *et al* (1959) did not find any autoregulation while others did (Weiss *et al* 1959) It might not be excluded that the anaesthesia influences on the ability of autoregulation

According to Table I and Fig 4 there is a reduction in the cardiac output when the aorta is clamped between the two renal arteries This reduction in the cardiac output was concomitant with a decrease in the total blood flow of the control kidney to the same extent but with apparently no redistribution in the cortical blood flow indicating a generalized increase in the renal vascular resistance From the present data nothing can be said regarding the location of that resistance change

The autoregulatory properties of the various parts of the renal parenchyma differ according to some authors (Thurau *et al* 1960) who found autoregulation weak or absent in the medulla Other authors however have not found any differences between the cortical and the medullary regions (Aukland 1966 Wolgast 1968 Grängsjö and Wolgast 1972 Löyning 1971) On the other hand the results of this investigation indicate large differences in the autoregulatory power of the inner and outer cortex The outer cortical zone did not show any autoregulation of the blood flow Zone 2 showed a slight autoregulation zones 3 and

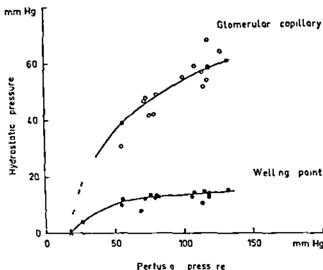


Fig. 5 The picture shows the hydrostatic pressures in the well ing points at different perfusion pressures The calculated values of glomerular capillary pressures are also represented in the figure The glomerular capillary pressure was calculated by adding 22 mm Hg (plasma oncotic pressure) to the stop flow pressures measured

4 did not only show an autoregulation but also an increased blood flow at a moderately decreased perfusion pressure These results agree well with those obtained by McNay and Abe (1970) who used the microsphere technique on dog kidneys The results of both these investigations indicate a vasoactive response to a decreased perfusion pressure which must be regarded as remarkable The heterogeneity of the vasoreactivity in the different zones of the cortex leads to a marked redistribution of the blood flow in favour of the inner parts of the cortex and also the medulla (Fig 3) through the true juxtamedullary glomeruli

In other investigations dealing with the autoregulatory problems the arterial blood pressure was increased by clamping the carotids with or without cutting the vagal nerves With this technique the arterial blood pressure and the renal perfusion pressure are increased but during the manoeuvre the sympathetic outflow may also be increased and it is plausible to assume an increased tone in the vasoconstrictor nerves to the kidneys The experimental situation thus implies both a vasoconstriction in the kidney and an increased perfusion pressure This is not comparable with the condition analyzed here In experiments on haemorrhagic shock Hellberg *et al* (1973) found a linear relation between the cardiac out

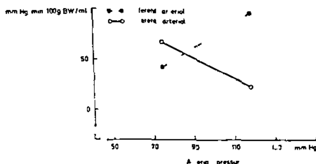


Fig. 6 The figure summarizes the resistance of the single afferent and efferent arterioles before the clamp ng and during the clamp ing

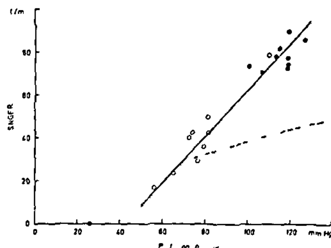


Fig 7 Single nephron glomerular filtration rate (SNGFR) versus the arterial perfusion pressure SNGFR is a calculated value according to the text The filled circles represent control normotensive conditions and the unfilled circles the values during the clamping The dashed line represents the SNGFR when an equilibrium does not exist at the end of the glomerular capillaries and is obtained according to the text

put and the total and the regional renal blood flow indicating a vasoactive response quantitatively the same in all parts of the cortical parenchyma—which is in agreement with the present findings (Fig 4)

The mechanisms behind the change in blood flow distribution in the autoregulating kidneys were analyzed in experiments where the hydrostatic pressures in the vascular structures on the renal surface were measured and related to the blood flow determined with the microsphere technique The results were very homogeneous and were regarded as being representative for nephrons having their glomeruli in the outer part of the cortex The results are not in accordance with the common opinion that the vasoactive response is restricted to the afferent arterioles but indicate that both the arterioles will be involved This opinion was also reached by Robertson *et al* (1972) using essentially the same approach

In 1966 Gertz *et al* measured the glomerular capillary pressure with the intratubular stop flow pressure technique and obtained glomerular capillary pressures within the range 85–90 mm Hg which values are very high when compared with the pressures of some 60 mm Hg found in this study The difference in the results depend probably on the fact that Landis technique used by Gertz *et al* has a tendency to give too high pressures Of interest is that their results showed an autoregulatory tendency in the glomerular capillary pressure in a similar way as found in this investigation Their constant capillary pressure in the hypertensive experiments must however partly be excluded from the discussion of autoregularoty mechanisms because of the dubious effect of carotid clamping—as discussed earlier

By multiplying plasma flow with a filtration fraction obtained from the effective filtration pressure (Landis and Pappenheimer 1963) a measure of the glomerular filtration rate in the superficial region can be calculated The hematocrit was assumed to be 50% and the protein concentration of plasma 6 % a complete filtration equilibrium *i e* the net driving forces equal zero is regarded to exist at the end of the glomerular capillary (Brenner 1971) The SNGFR of the normotensive animals were according to Fig 7 calculated to be about 80 nl/min This value is reasonably too high in comparison with direct measurements and the

discrepancy between the calculated and expected values seems most likely to depend on the erroneous assumption of a filtration equilibrium at the end of the glomerular capillary In a forthcoming paper (Kallskog *et al* 1975) direct measurements of the glomerular filtration rate and the single glomerular plasma flow are presented and the existence of a disequilibrium is predicted Values from that study would give an autoregulation of the SNGFR, shown in Fig 7 as the dashed line

The physiological significance of the different autoregulatory properties of the glomerular populations is somewhat unclear Barger (1973) has reviewed the literature and made the assumption of outer salt loosing nephrons and inner salt saving nephrons The first indications of such a possibility were suggested by Horster and Thurau (1968) and Gertz *et al* (1969) in investigations showing a rearrangement of the glomerular filtration At high sodium intake the GFR rose in the outer glomeruli and decreased in the inner ones while the opposite happened at low sodium intake The diversion of blood from the inner salt saving nephrons to the outer salt loosing nephrons when the blood pressure is increased might then explain the nearly linear relationship between the arterial blood pressure and the urine flow at an arterial blood pressure above some 50 mm Hg.

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A Theoretical Study of Restricted Convection-Diffusion as Applied to Blood-Tissue Barrier Exchange

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Received 7 November 1974

Abstract

ÅBERG B *A theoretical study of restricted convection–diffusion as applied to blood-tissue barrier exchange* Acta physiol scand 1975 *94* 301–308

This paper handles a model of the capillary function in the exchange of uncharged molecules between the blood and the tissue The capillary system is subdivided into a filtering and a reabsorbing part. The exchange is assumed to occur through channels which are described in operational terms as pores. Through these pores there is a transport of solutes by concomitantly acting convection and diffusion influenced by a steric hinderance (restricted convection-diffusion) The outflux of glucose and raffinose is enhanced in the filtering pores raffinose relatively more than glucose In the reabsorbing pores the outward diffusion is hindered to some extent raffinose relatively more than glucose It is shown that the net effect of filtration and reabsorption is to increase the outflux of raffinose as compared to that of glucose This mechanism may explain why glucose and raffinose and other small molecules appear to pass across capillary walls in proportion to their free diffusion coefficients and not in proportion to their restricted diffusion coefficients.

The transport of neutral solutes across capillary walls is generally assumed to occur through pores (opened intercellular junctions *cf* Karnovsky 1970) In experiments where the plasma concentration of dextran molecules of different sizes were held constant and plasma and lymph concentrations were compared the average pore radius could be estimated to about 4 nm (*cf* Grotte 1956) The results indicated that low molecular weight dextrans passed out of the capillaries in proportion to their restricted diffusion coefficients A restriction due to steric hinderance should also affect the outflux of such molecules as glucose and raffinose through the capillary wall (Beck and Schultz 1970) but such a restriction could not be found in in vivo experiments with single injection technique (Crone 1963 Kampp 1970) In a series of in vitro experiments with rather high precision it could be verified that the outflux of raffinose and glucose indeed seemed to occur without restriction due to steric hinderance in pores (Åberg 1974)

In his model of the capillary system Starling (1896) proposed that there is filtration of fluid in the arterial part of the capillary and reabsorption in the venous part A fluid filtration may oppose the hinderance exerted by steric restriction in a single pore (Åberg

Hågglund 1974) The aim of this communication was to evaluate if simultaneous convection and diffusion may give outflux rates of low molecular weight solutes in single-injection experiments that are proportional to the free diffusion coefficients rather than to the restricted diffusion coefficients

The Model

Restricted convection diffusion

The transport of neutral molecules through pores in the capillary wall is considered to occur by concomitant diffusion and convection The word pore is here used as an operational term If J_s is the solute flux density $c(x)$ is the concentration along inside of the pore v the mean linear fluid flow velocity and D the free diffusion coefficient then the flux density (mass flow velocity per unit pore area) is calculated as

$$J_s = -k \cdot D \frac{\partial c(x)}{\partial x} + k \cdot v \cdot c(x) \tag{1}$$

where k is a conductance coefficient assumed to be valid for both diffusional and convectional transport. This coefficient is equal to the ratio between the apparent pore area per 100 g tissue (A') for the solute and the pore area per 100 g tissue (A_p) It has generally been related to the molecular and the pore dimensions (Bean 1972)

$$k = A'/A_p = [2(1-a/r)^2 - (1-a/r)^4] \cdot g(a/r) \tag{2a}$$

$$g(a/r) = 1 - 2a^2/3r^2 - 0.162(a/r)^5 - 0.406(a/r)^7$$

where a = molecular radius and r = pore radius

The free diffusion coefficient D is assumed to be satisfied by the Stokes Einstein relation

$$D = RT/(6\pi \cdot \eta \cdot N \cdot a) \tag{3}$$

where R is the gas constant T is absolute temperature η is viscosity and N is Avogadro s number

With proper choice of test substances the conditions within the pore can be approximated by a steady state transport (build up time of the concentration profile is short as compared to transport time Åberg and Hägglund 1974 Åberg 1974) Eq (1) is then solved by integration putting $c(x) = c_0$ for $x = 0$ (plasma side) and $c(x) = c_d$ for $x = d$ (tissue side)

$$J_s = k \cdot v \frac{c_0 - c_d \cdot \exp(-v \cdot d/D)}{1 - \exp(-v \cdot d/D)} \tag{4}$$

The concentration profile within the pore is unaffected by k as k is assumed to have the same influence on both the convectional and the diffusional transport (cf Forster 1971)

Model of the capillary

The capillary system is subdivided in two main parts one with filtrative and the other with reabsorptive properties In the filtering or arterial part of the capillary system the total pore area per 100 g tissue is A_{p1} the linear flow velocity through the pores v_1 and the corresponding solute flow $J_{s1} \cdot A_{p1}$ In the reabsorbing part the total pore area per 100 g tissue is A_{p2} the linear bulk flow through the pores v_2 and the solute flow $J_{s2} \cdot A_{p2}$—all parameters assumed operational model averages

In the general case the solute balance is given by

$$J_{s1} \cdot A_{p1} + J_{s2} \cdot A_{p2} - L \cdot c_d = 0 \tag{5}$$

where L = lymph volume flow The corresponding fluid balance is governed by

$$v_1 \cdot A_{p1} + v_2 \cdot A_{p2} + F - L = 0 \tag{6}$$

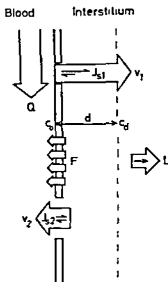


Fig 1 Model of the capillary The capillary system is reduced to a single capillary with filtering and reabsorbing parts. In the filtering part the linear velocity of the fluid flow is denoted as v_1 and in the reabsorbing part as v_2 In constant concentration experiments the capillary and tissue fluid concentrations are assumed to be in balance When the concentration on the tissue side of the transport path d is zero (single injection type of experiments) there is a net flux of solute from the capillary to the tissue (J and J_{s2}) Q is blood flow L lymph flow and F is flow of fluid free from the test solute

where F is the reabsorption of fluid free from the test solute (Fig 1) F is introduced mainly to balance Eq (6) It may be seen as a solvent flow from the tissue to the blood through small pores or through the endothelial cells not available to a particular solute as discussed by Yudilevich and Alvarez (1967) Ussing (1970) and Tosteson (1970)

Application of the convection–diffusion model to single injection technique

In the single injection technique the transport of solutes across the blood tissue barrier is expressed in terms of extraction (E) this is a measure of the loss of solute into the tissue as compared to the amount of solute injected into the capillary As the solute loss is solute flux density times transport area and amount injected is plasma concentration times plasma flow a general formulation of extraction is

$$E = \frac{A_p \; J \; (t)}{Q \; c \; (t)} \tag{7}$$

where Q is plasma flow per 100 g tissue and c (t) the concentration in the capillary

The solute flow J (t) is proportional to the momentary concentration $c_a(t)$ (Åberg and Hägglund 1974) and the extraction is consequently time invariant

$$E = \frac{A_p \; J}{Q \; c} \tag{7a}$$

In the situation in which the tissue concentration $c_d = 0$ the following equation is obtained from Eqs (4) and (7a)

$$E = \frac{k_1 \; v \; A}{Q\{1 - \exp\;(-v \quad d/D)\}} \tag{8}$$

where E is the extraction in the arterial part of the capillaries and k_1 is the conductance factor (Eq 2) in the filtering pores In the venous part, the plasma concentration of the solute is $(1 - E)$ c_a. The passage of solute within the capillary is assumed fast as compared to the longitudinal tissue diffusion The tissue concentration is accordingly still assumed to be zero This assumption is justified if the extraction is during the passage of the major part of the bolus (Åberg 1974) The venous extraction E_2 is thereby

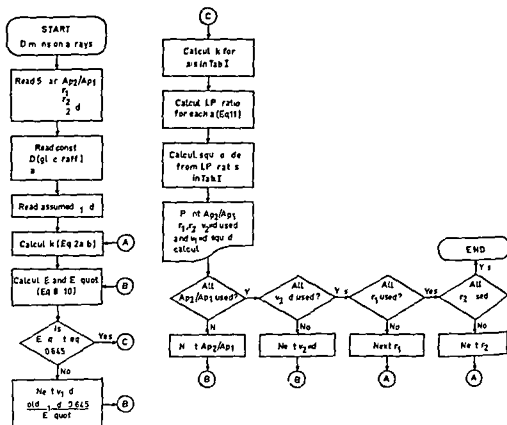


Fig 2 Flow diagram of calculations The variables are inserted in arrays of 5 members Each possible combination of the members is used for calculation of the 5th variable v_1 d (left column) With the aids of all five variables the function of the lymph to plasma concentration ratio (LP ratio) against the diffusion radius (a) is calculated and compared by the sum of square deviations to the experimental values of Grotte (1956) When all 625 results are obtained the minima of the square deviations are studied by new sets of variables with smaller increments

$$E_2 = \frac{k_2 \; v_2 \; A_{p2} \; (1 - E_1)}{Q\{1 - \exp\;(-v_2 \; d/D)\}} \tag{9}$$

where k_2 is the conductance factor in the reabsorbing pores Now the extraction may be assumed to occur first in the filtering capillaries and subsequently in the reabsorbing capillaries The combined extraction E is then

$$E = E_1 + E_2 \tag{10}$$

Application of the restricted convection diffusion mod l to the constant concentration technique

In the constant concentration technique the permeability characteristics are derived from the LP ratios i e lymph (or tissue fluid) concentration (c_d) over plasma concentration (c_p) c_d/c_p may be solved in terms of the transport parameters by combining Eqs (4) and (5)

$$\frac{c_d}{c_p} = \frac{k_1 \; v_1 \; A_{p1}/(1-p) + k_2 \; v_2 \; A_{p2}/(1-q)}{k_1 \; v_1 \; A_{p1} \; p/(1-p) + k_2 \; v_2 \; A_{p2} \; q/(1-q) + L} \tag{11}$$

where $p = \exp\;(-v_1 d/D)$ and $q = \exp\;(-v_2 d/D)$

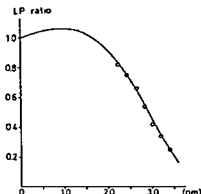


Fig 3 Best fitted parameters Lymph to plasma concentration ratio (LP ratio) for different molecular radia (a) theoretically with the best fitted values of the parameters in Tab I (continuous line) compared to the experimentally found values (circles)

Numerical Calculations from Model Concepts

General assumptions

In order to obtain a rough estimation of the transport parameters involved, the data from single injection experiments by Åberg (1974) and continuous infusion experiments by Grotte (1956) are used The total pore area ($A_{p1}+A_{p2}$) is assumed to be 1 4 cm^2/100 g tissue (Karnovsky 1970) and the transport path length d is set to 10 μm which includes an equivalent path through unstirred layers

Albumin is known to pass the capillary walls with difficulty Assuming reabsorption at the venous end of the capillaries which probably is more permeable than the arterial end (Smith and Rouś 1931 Hauck and Schroer 1969) although still not permeable to proteins to a visible extent (Wiederhielm 1968) the operational reabsorption pore radius ought not to be larger than about 6 nm Values between 4 and 6 5 nm are tested in the model

The extraction quotient raffinose over glucose is set equivalent to the ratio of their free diffusion coefficients 0 645 The remaining parameters A_{p2}/A_{p1} r_1 r_2 v_1 and v are solved in a trial and error procedure in the following manner (Fig. 2) A_{p2}/A_{p1} r r and v_2 d five values of each are inserted into Eqs (8)–(10) whereby 625 values of v_1 d are obtained each value corresponding to one value of the other parameters Those sets of values which fit best to the 6 LP ratios of Grotte inserted to Eq (11) are then varied within smaller limits again in Eqs (8)–(10) until the best possible visual fit is obtained The difference of v A_{p1} and v_2 A_{p2} is a flow of water in routes other than through pores (lymph and through endothelial cells or tight junctions") here noted $L+F$

This fitting shows that if A_{p2}/A_{p1} is too small i e reabsorption through pores is small the reabsorption through cells becomes large and the extravascular molecules will be too much concentrated in constant concentration experiments The same will be the case if the reabsorption flow v is too small The effect of varying r_1 and r of course is to set the limits for the passage of the molecules

Results

The results of the mathematical procedure in order to correlate the model to the experimental data are shown in Fig 3 where the results of the final best visual fit was obtained The best fitted set of parameters are listed in Table I

The influence of the parameters on the model behaviour is shown by varying them ±1(

TABLE I Input to and output from the numerical calculations on the model

Constants used

1 Single injection situation
Mean plasma flow = 0 188 ml/s
Mean tissue weight = 250 g
Extraction ratio raffinose/glucose = diffusion coeff ratio = 0 645

2 Constant concentration situation (Grotte 1956)

Diffusion radius (nm)	2 22	2 42	2 64	2 83	3 01	3 20
LP ratio	0 82	0 75	0 66	0 54	0 42	0 34

Mean lymph flow (L) = 7 10 $^{-8}$ ml/s and 100 g tissue (estimated)

Best fitted variables to the constants used

$v_1\ d$ = 0 26 10 $^{-8}$ cm²/s	$v_2\ d$ = −0 16 10 $^{-8}$ cm²/s
r_1 = 3 9 nm	r_2 = 5 1 nm
d = 5 μm	A_{p2}/A_{p1} = 1 15

keeping the others constant at their calculated values The result is shown in Fig 4 a e This model is relatively more sensitive to parameter variations in the constant concentration situation (continuous lines) than in the transient concentration (single injection) situation with smaller molecules (figures in the upper right corners) It is most sensitive to variations in the pore diameters and for that reason these parameters seem to be those which are best determined

Discussion

The magnitude of the parameters

A calculation of the filtration flow velocities from a measure of the relative movement of red blood cells with the microocclusion technique has been done by Zweifach and Intaglietta (1966) The fluid movement from the capillary to the tissue ranged from 0 119 to 0 070 μ³ μ⁻² sec⁻¹ Assuming again that 0 02 per cent of the vessel area consists of opened intercellular junctions and that d is 5 μm the values of Zweifach and Intaglietta can be recalculated to a $v\ d$ of 3 10⁻⁴ to 0 5 10⁻⁴ cm²/sec which very much exceeds the values of 0 26 10⁻⁸ to 0 16 10⁻⁸ cm²/sec calculated in this work

The sum of forces needed to exert a fluid filtration velocity times diffusion path length

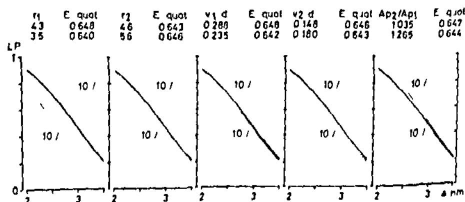


Fig 4 Parameter variations The effect of a 10 variation of one of the parameters in Fig (11) r_1 and r_2 are given in nm v_1 d and v_2 / in cm²/sec 10⁸ The effect on extraction quotients (E quot) are small The model is most sensitive to variations in pore radii

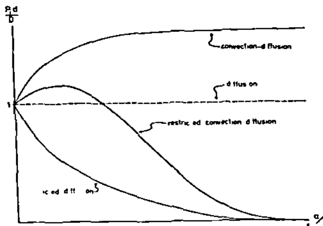


Fig 5 Comparison between d fferent transport mechanisms The permeability of a substance i (P_i) per its free diffusion coefficient (D_i) per transport distance (d) is constant in unrestricted diffusion. In convection–diffusion it reaches a plateau where the diffusional part is negligible (has no meaning here as transport is unrestricted *a*/*r* is to be seen as a molecular d mension only) In restricted diffusion the permeability falls with increas ng ratio of the molecular (*a*) to the pore (*r*) radius. In restricted convection–diffusion the permeab l ty increases is constant, or decreases depending on the convection flow velocity and the ratio *a*/*r*

of 0 26 10^{-6} cm²/sec through a pore with a radius of 3 9 nm is hard to evaluate If the law of Poiseulle was valid the force needed should be about 3 5–4 kPa (25–30 mm Hg) if the thickness of the capillary wall is about 0 5 μm but if the restriction is represented by a short distance along the pore the force needed for *water* propagation is less and within reasonable values for the transmural pressure in the arterial parts of the capillary (The constriction effect Perl 1971)

On the other hand the flow velocity might be underestimated Restriction is calculated according to an empirical formulation emanating from works by Faxén (1922) and Ferry (1936) Renkin has used this restriction formulation in a somewhat different way (Renkin 1954) and if his formulation is used the filtration flow velocity must be increased about 5 fold As an intermediate Verniory *et al* (1973) used different formulations for restriction of the molecules if they were subjected to a chemical or a velocity gradient across the glomerular wall In those cases the ordinary Starling forces might not be sufficient

Concluding remarks

In single injection experiments smaller molecules are transported across the walls of the muscular capillaries as if there was no restriction whereas in constant concentration experiments the restriction of larger molecules is obvious In Fig 5 the general behaviour of restricted convection-diffusion transport is described in comparison to diffusion and convection–diffusion without restriction and the restricted diffusion in terms of permeability per transport path length for different molecular sizes Transport by a restricted diffusion should be substantially hampered already at small ratios of molecule/pore radius which seems not to be the case Restricted convection-diffusion meets with no apparent restriction to smaller molecules but with restriction to the larger ones The length of the apparently

unrestricted part (LP ratio about unity in Fig 3 permeability equal to or larger than D/d in Fig 5) and the slope of restriction can be varied by the model parameters The fitting of this model to experimental results can be done with reasonable values of these parameters

Thanks are due to the entire staff of the Institute of Physiology and Medical Biophysics for valuable discussions The author also wishes to thank Mrs Ulla Britta Berg and Helena Billander for technical assistance in the drawings and calculations

The work was supported by grants from the Medical Faculty of the University of Uppsala the Swedish Research Council (Project No 14X 629) and the National Institutes of Health (Grant No 5 R01 HE 12960-08)

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Acta physiol scand 1975 94 309–312

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The Effect of Restricted Convection–Diffusion on Bolus Concentration along an Exchange Vessel

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Received 7 November 1974

Abstract

Åberg B *The effect of restricted convection–diffusion on bolus concentration along an exchange vessel* Acta physiol scand 1975 *94* 309–312

If a sudden rise in solute concentration travels through a capillary system a diffusible neutral solute passes the walls of the exchange vessels If then the rate of passage depends on a chemical potential gradient (diffusion) and a hydrodynamical potential gradient (convection) and is sterically restricted (restricted convection–diffusion) water and low molecular weight solvents leave the filtering part of the capillary at a higher rate than the solute causing a relative increase in its concentration. This phenomenon and the effect of an increasing pore size along the capillary is investigated in a model for an impermeable solute and two diffusible solutes corresponding to glucose and raffinose It is shown that in the model the concentration fall along the capillary tends to become rectilinear

The transport of uncharged molecules across capillary walls might be calculated from the disappearance of a bolus injected into the capillary system In this case a longitudinal intracapillary concentration profile of the substance must be assumed The most often used assumption is that the concentration falls exponentially along the exchange vessel (Crone 1963 Goresky *et al* 1970) This assumption is valid if there is no flow of water across the blood tissue barrier and if the permeability is constant along the capillary but this does not seem to be the case (*cf* Hauck 1969)

In an earlier work (Åberg 1974 b) it has been pointed out, that the chemical potential can not alone give rise to the experimentally found transport rates of smaller molecules across the capillary wall if the transport is considered to occur through sterically restricting pores If there is however an additional convection transport the effect of restriction on the smaller molecules can be counteracted

The introduction of a filtration and a reabsorption as well as a gradient of permeability along the capillary affects the concentration along this vessel The aim of this communication was to study the concentration decrease along an exchange vessel in a simple mathematical model having larger pores towards the venous side with a filtration from lary to the tissue on the arterial side and a reabsorption on the venous side

Method

The calculation was made with a finite difference method. The capillary length was divided in sections, the concentration in each section calculated as a result of water and solute flows across the wall and the blood flow and the concentration in the preceding section.

Assumptions

The pore radius (r) is assumed to increase linearly along the length (l) from entrance to exit of the exchange vessel

$$r(l) = r(o) + h \cdot l \tag{1}$$

where h is a constant.

The convection flow times the transport distance (v d) is assumed to depend on pore radius $(r(l)/r(o))^2$ and a net driving force. This driving force tends to be relatively complex even in a simple model like this. The pressure drop along a channel with porous walls is not rectilinear but larger in the beginning than at the end even if the wall porosity is homogeneous (Berman 1953). Also the osmotic force might decrease in a non-rectilinear fashion depending on the decreasing reflection coefficient for the permeable molecules. For the sake of simplicity the net driving force is assumed to fall exponentially along the capillary. This force is positive in the filtering parts, negative in the reabsorbing parts and zero at the point of balance (l_b)

$$v(l)\,d = v(o)\,d\,\{\exp(-k\,l) - \exp(-k\,l_b)\}\{(r(o) + h\,l)/r(o)\}^2 \tag{2}$$

where k is a constant.

The solute flux (J_s) across each section is assumed to occur by convection diffusion (Åberg and Hesslund 1974). The intracapillary concentration is assumed to be radially homogeneous (c) and the concentration on the tissue side of the transport distance (d) is assumed to be zero. (Extraction is linear over the major part of the bolus as in the case of raffinose and glucose, Åberg 1974)

$$J_s(l) = v(l)\,\Delta Ap\,q(l)\,c(l)/\{1 - \exp(-v(l)\,d/D)\} \tag{3}$$

where ΔAp is the pore area of each section, D is the free diffusion coefficient and q is a constant factor related to the pore radius (r) and the molecule radius (a)

$$q(l) = \{2(1-b)^2 - (1-b)^4\}\{1 - 2.104\,b + 2.09\,b^3 - 0.95\,b^5\} \tag{4}$$

where $b = a/r(l)$

Numerical calculations

The model parameters are given in Table 1, obtained from earlier calculations (Åberg 1974 b). The vessel is divided into 43 sections of which 20 are filtering and 23 are reabsorbing. (Reabsorbing area/filtering area = 1.15.) At the entrance of the exchange vessel the concentration is set to unity ($c(o) = 1$).

TABLE 1. Input parameters to the model. The magnitude of the parameters are the best fitted to a restricted convection diffusion model taken from an earlier work (Åberg 1973).

Parameter	Value
Mean filtration flow velocity · transport distance =	0.26 · $10^{[illegible]}$ cm²/s
Mean reabsorption flow velocity · transport distance =	0.1[illegible] · $10^{[illegible]}$ cm²/s
Mean filtering pore radius	3.[illegible] nm
Mean reabsorbing pore radius =	5.1 nm
Plasma flow/100 g tissue =	0.075 cm³/s
Ratio filtering reabsorbing area =	1.15

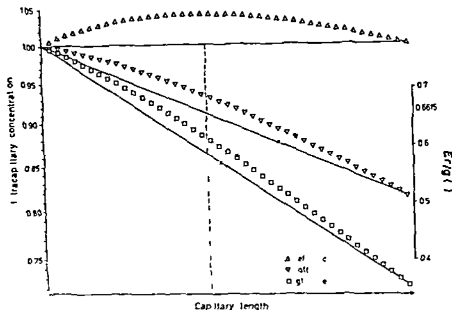


Fig. 1 Results of calculations The exchange capillary is divided into 43 sections and the concentration of a passing bolus is calculated in each section for a reference substance and raffinose and glucose as a result of restricted convection-diffusion transport across the capillary wall The concentrations are plotted on a logarithmic scale (left scale) and the build up of an extraction quotient raffinose/glucose (Er/g) can be followed (right scale)

Assume i and j to be two consecutive sections Q is the plasma flow and ΔF is the trans-endothelial flow free from the solute investigated in each section Then

$$Q(j) = Q(i) - r(i) \times \Delta Ap - \Delta F \quad (5)$$

$$C(j) = [Q(i) \times C(i) - J(i)]/Q(j) \quad (6)$$

From Table I h and $r(o)$ are easily calculated to 0 0558 nm/l and 3 342 nm respectively ΔAp was set to 1 4/43 = 0 0326 cm²/100 g tissue (Total capillary area = 7 000 cm /100 g tissue whereof 0 02° consists of opened intercellular junctions Karnovsky 1970) and d was set to 5 μm

k and $r(o)$ d has been calculated by iterations from Eq (2) so that $[\Sigma^{2} r(l) \ d]/20 = 0$ 26 10 ⁶ cm /sec and $[\Sigma_{20}^{3} r(l) \ d]/23 = -0$ 16 10⁻⁶ cm²/sec

Results

The results from the calculations of 3 different molecules are shown (Fig 1) one ideal reference ($a \sim 10$ nm) and two diffusible molecules ($D = 0$ 55 10⁻ cm /s $a = 0$ 61 nm corresponding to raffinose and $D = 0$ 85 10⁻ cm /s $a = 0$ 39 nm corresponding to glucose) The exponential concentration fall is represented by a straight line The concentration fall of glucose happens to be nearly rectilinear and that of raffinose is more convex

Discussion

A bolus of neutral solutes is supposed to pass an exchange vessel When there is a convective flow of water to the tissue and the solute transport across the capillary wall is relatively more restricted than the water transport the solutes remaining inside the capillary are more concentrated than without that convection flow Calculating with an exponential fall in the concentration would in this case give rise to a slight underestimation of the chemical potential gradient across the blood–tissue barrier

In an earlier work (Åberg 1974 b) the transport calculations were simplified by taking the arterial concentration as the chemical driving force to the balance point l_b, from where the corresponding force was set equal to the venous concentration This calculation in steps obviously led to an overestimation of the arterial driving force and an underestimation of the venous driving force The combined effect of this can be evaluated from the present results. Starting with an extraction quotient of 0 645 the parameters used in the present calculations were estimated. Here the calculations are made "the other way around" which leads to about the same extraction ratio 0 662.

In conclusion a flow of water from the exchange vessels to the tissue has a twofold increasing effect on the solute transport (a) to give an additional convectional transport from the blood to the tissue and (b) to increase the chemical activity of solutes more restricted than water

I wish to express my thanks to C. A. Goresky for initiating this work

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Acta physiol scand 1975 94 313–318

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Enzyme Activities and Muscle Strength after "Sprint Training" in Man

By

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Received 15 November 1974

Abstract

Thorstensson A B Sjödin and J Karlsson *Enzyme activities and muscle strength after sprint training in man* Acta physiol scand 1975 *94* 313–318

Sprint type strength training was performed 3–4 times a week for 8 weeks by 4 healthy male students (16–18 yrs) The training was carried out on a treadmill at high speed and with high inclination Muscle biopsies were obtained from vastus lateralis before and after the training period for histochemical classification of slow and fast twitch muscle fibres and for biochemical determination of metabolites and enzyme activities Muscle fibre type distribution was unchanged whereas fibre area indicated an increase for both fibre types in 3 subjects after training The muscle enzyme activities of Mg^{2+} stimulated ATPase myokinase and creatine phosphokinase increased 30 [illegible]0 and 36 percent, respectively Muscle concentration of ATP and creatine phosphate (CP) did not change with training Sargent s jump increased with on average 4 cm (from 47 to 51 cm) maximal voluntary contraction (MVC) with 19 kp (from 165 to 184 kp) and endurance at 50 percent of MVC with 9 s (from 47 to 56 s) respectively After training all subjects showed a gain in body weight (mean 1 4 kg) and in thigh circumference (mean 1 5 cm) indicating a larger leg muscle volume and consequently also an increase in total ATP and CP

High intensity exercise of short duration will apply high demands on the immediate energy supplying mechanisms in the muscle and repeated exercise of this type has been shown to cause exhaustion of phosphagen stores in the exercising muscles (Karlsson and Saltin 1971) Animal studies have demonstrated that short term high intensity training programs cause an increase in creatine phosphokinase activity (Staudte Exner and Pette 1973) and an increased storage of creatine phosphate has been indicated after sprint training of rats at high altitude (Gale and Nagle 1971)

The aim of the present study was to investigate quantitatively and qualitatively the effects of a high intensity intermittent sprint training program on skeletal muscle in man Special interest was focused on the turn-over mechanisms of ATP by examining the activities of actomyosin ATPase (E C 3 6 1 3) creatine phosphokinase CPK (E C 2.7 3 2) and myokinase MK (E C.2.7 4 3) as well as the size of the phosphagen stores before and after the training period In addition histological analyses were performed to evaluate changes in muscle fibre type composition

Material and Methods

Subjects 4 healthy moderately trained male students (16–18 yrs) participated in the study Although the subjects were in late adolescence growth effects are assumed to be insignificant due to the short observation period No increase in body height was observed during the period

Training procedure The subjects were training 3 to 4 times a week over a period of 8 weeks. The training bouts were performed as short term intermittent sprint training (5 s running) on a motordriven treadmill at high speed and with steep inclination The rest periods between each bout were 25–55 s Over the training period the speed inclination and number of repetitions were successively increased from on average 19 km/h 9 and 20 repetitions per bout to 24 km/h 10 and 40 repetitions per bout The basis for these gradual increases in training load and intensity was to force the subjects to approximately the same level of exhaustion after each training session as the training program proceeded and the capability of the subjects improved

Although sprint training from a practical point of view is a well known procedure it was thought of interest to further define the protocol applied Therefore V_{O_2} HR and blood lactate concentration were determined at appropriate intervals during one training session (subject H O 100 × 5 s speed 24 km/h, inclination 11 rest periods 55 s) using standard procedures employed in our laboratory (see e g Christensen *et al* 1960)

All tests and analyses were carried out before the start of the training and 2 days after the termination of the training period according to the same protocol

Functional and anthropometrical tests The dynamic strength in the legs was assessed by means of Sargent s jump i e vertical jumps from a squatted position and by Margaria test (Margaria Aghemo and Rovelli 1966) i e running at maximal speed in a staircase with 36 cm step height and 33 inclination The isometric leg strength was measured as maximal voluntary isometric contraction (MVC) and as endurance time at 50 of MVC (Karlsson and Ollander 1972) Sprint acceleration tests over 25 m were performed using conventional sprint starting positions The timing in the sprint and in the Margaria test was done with 0 001 s accuracy on a digital timer (TF 2414/1 Markoni Instrument Ltd) Individual maximal oxygen uptakes (V_{O_2} max) were determined on a bicycle ergometer Expired air was collected in Douglas bags and gas analyses were carried out on a Haldane apparatus (Åstrand and Saltin 1961)

To evaluate gross anthropometrical changes due to the training body weight height, and thigh circumference of both legs were determined Thigh girth was measured in a horizontal plane just below the lowest point in the gluteal furrow

Biochemical and histochemical analyses Muscle biopsies were obtained from the lateral portion of the thigh (m vastus lateralis) (Bergstrom 196) The biopsies for the metabolite and enzyme activity analyses were immediately frozen in liquid nitrogen (within 3–4 s) and stored at −80 C The enzyme activities of Mg^{2+} stimulated myofibrillar ATPase CPK MK and lactate dehydrogenase LDH (E C 1 1 1 7) as well as the concentrations of ATP and creatine phosphate (CP) in the muscle were determined according to the Lowry techniques (Lowry and Passonneau 1972) as modified in our laboratory by Karlsson (1971) The biopsies for the histochemical studies were mounted and frozen in isopentane Sections of 10 µm were cut and stained for identification of slow twitch (ST) and fast twitch (FT) muscle fibres and estimation of fibre area (for ref see Gollnick *et al* 1972)

To assess fibre recruitment pattern during this extreme type of exercise selective glycogen depletion was evaluated by means of PAS staining (Gollnick *et al* 1974 a) of biopsy specimens obtained before and after the training session described above (subject H O)

Statistical methods Ordinary statistical methods were applied for calculation of mean values and standard error of the means Intraindividual differences between values before and after training were tested by the Student s t test. In testing hypothesis one-tailed tests have been used (Ferguson 1966 Siegel 1956)

Results

The results from the single training session (Fig 1) are in general agreement with what has previously been reported for similar intermittent exercise (Christensen *et al* 1960 Saltin and Essen 1971) V_{O_2} during activity averaged 2 8 l/min or approximately 70% of V_{O_2} max

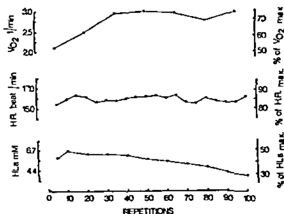


Fig 1 Oxygen uptake heart rate and blood lactate concentration for subject H O performing 100×5 s sprint running (rest intervals 55 s) with a speed of 24 km/h and an inclination of 11 presented in absolute figures and in percent of maximal values.

whereas the corresponding value for the rest periods was 2 0 l/min Blood lactate after every 10th run ranged 4 3–6 7 mM with a mean of 5 6 mM The relatively high lactates might be related to the fact that the exercise intensity in the present experiment exceeded that applied by *e g* Saltin and Essén (1971)

Both ST and FT fibres showed glycogen depletion after the training session This indicates that both fibre types were recruited to produce muscle tension during the sprint type exercise performed in the present study

All subjects demonstrated increases in body weight (mean value 1 4 kg) after the training period whereas no changes in height were observed (*Table I*) *Simultaneously* there was an increase in thigh circumference with on average 1 5 cm (Table I) This indicates that the increase in body weight at least partly was attributable to an augmented leg muscle mass Increases in MVC Sargent s jump and endurance at 50 MVC were demonstrated for all subjects whereas 3 subjects improved their performance in the Margaria and the sprint tests (Table II) Consequently the sprint training program improved not only dynamic but also isometric performance The relative distribution of fibre types was not altered as a result

TABLE I Anthropometrical and histological measurements and muscle phosphagen concentrations before and after 8 weeks of sprint training, and intraindividual differences (d) presented as means and standard error of means (S E.) and the probability (p) of finding a t value more extreme than the observed under the null hypothesis (ns = p > 0 1) (n = 4)

	Before	After	d	p
Body weight, kg	66 4±3.0	67 8±3 1	1 4±0..	<0 01
Body height, cm	179 0±3 67	179 1±3 63	0 1±0 13	ns
Thigh girth cm	53 0±1 60	54 5±1 11	1 5±0 51	<0 05
FT	59 ±7 3	56 ±7 4	3 ±2.1	ns
Fibre area μm^2 ST	5 060 ±.33 4	5 190 ±183 8	130 ± 44 0	ns
FT	5 500 ±283.	5 740 ±316.1	240 ±446 4	ns
V_{O_2} max ml kg⁻ × min	53 7±2.05	55 4±.35	1 7±1 18	ns
ATP mmol g⁻	5 1±0 30	5 4±0.6	0 3±0 2.	ns
CP mmol g^{-1}	19 9±0 7?	17 5±0 59	.4±1 75	ns

TABLE II Functional tests before and after 8 weeks of sprint training presented as in Table I (n = 4)

	Before	After	d	p
MVC kp	165±11 5	184±20 6	19 0±9 21	<0 1
50 MVC sec	47±3 4	56±2 4	9±3 78	<0 05
Sargent s jump cm	47±2 3	51±1 6	4±1 36	<0 05
Margaria test kpm × s^{-1}	107 9±3 98	112.5±3 25	4 6±2 32	<0 1
25 m sprint sec	3 78±0 079	3 75±0 063	0 03±0 018	<0 1

of the training (Table I) which is in accordance with other training studies on endurance (*Gollnick et al 1973*) *as well as strength training (Thorstensson and Karlsson 1974) Fibre* areas of ST as well as FT fibres on the other hand indicated increases in 3 subjects which might suggest that the leg muscle hypertrophy presented above was related to training induced growth of both ST and FT fibres Although the muscle concentrations of ATP and CP remained unchanged as compared to before training (Table I) the total amount of phos phagens was increased as a result of the augmented muscle mass Training effects were demonstrated in the activities of the selected high energy phosphate transferring enzymes (Table III) Increases corresponding to 30 36 and 20% were shown for Mg^{2+} stimulated ATPase creatine phosphokinase and myokinase respectively

Discussion

The major finding in the present study was the qualitative changes in the muscle tissue as pressed by the increases in enzyme activity The significance of these differences were of the same order as for the improvements in muscle performance In addition it seems reason able to suggest muscle hypertrophy based on the increased leg muscle circumference Wheth er this was related to an increased fibre area cannot be concluded but seems reasonable

In the present study no attempts were made to separate the protein complexes involved in the contraction machinery as *e g* described by Gergely (1968) and as recently presented by Taylor Essén and Saltin (1974) for purified myosin The reason for this was that any similar separation procedure would exclude the possibility of examining the complete protein system of the contraction apparatus of the myofibril Mg^{2+} stimulated ATPase activity as *determined in the present study is considered to be a representative measurement* of actomyosin ATPase activity in the muscle (Ebashi 1972) The demonstrated increase in the

TABLE III Enzyme activities before and after 8 weeks of sprint training presented as in Table I (n = 4)

	Enzyme activity moles 10^{-4} g^{-1} min^{-}			
	Before	After	d	p
Mg^{2+} ATPase	0 070±0 0046	0 091±0 0051	0 0?1±0 0088	<0 05
MK	1 86 ±0 .38	2 .3 ±0.231	0 37 ±0 134	<0 05
CPK	0 99 ±0 150	1 35 ±0 151	0 36 ±0..19	<0 1
LDH	1 56 ±0 68	1 66 ±0 .8.	0 10 ±0 351	ns

activity of this enzyme would consequently be an acceptable indicator of changes in con tractility on the myofibrillar level due to the training Taylor Essen and Saltin (1974) presented a relationship between Ca^{2+} stimulated myosin ATPase activity and muscle fibre composition which gave no or very little space for the possibility of training effects on myosin ATPase activity according to the authors conclusion An explanation to our find ings on the basis of changes in the individual proteins involved in the muscle contraction considering the suggested unchanged myosin ATPase activity cannot be given at the pres- ent stage of these studies in progress

In addition to Mg^{2+} stimulated ATPase activity MK and CPK activities have been deter mined to give as complete a description as presently possible of the enzymes most probably of significance in the immediate turn-over of ATP quanta in relation to sprint type exercise The rational for this would be that these enzymes are catalyzing reactions replenishing ATP from ADP (MK) and CP (CPK) respectively A biological implication of our findings of increased MK and CPK activities with training would then be to enhance ATP resynthesis for extreme muscle activities such as sprint type exercise

Whether the resynthesis of ATP from ADP and CP is faster than the ATP regeneration from the anaerobic glycolytic pathway is at present uncertain LDH activity was examined as a potential marker of the anaerobic glycolysis (Gollnick *et al* 1974 b) but no enzyme activity changes due to the training could be detected (Table III)

As already pointed out the subjects were in late adolescence and consequently the possi bility of growth during the training study has to be considered No changes in height were however present The observed changes in leg muscle circumference as well as strength parameters seem then to be related to the training procedure rather than growth and/or maturation To which extent the changes in muscle strength were due to the suggested in creases in muscle mass or to the qualitative changes in terms of enzyme activities or both has to be the subject for further investigations

This study was supported by grants from the Swedish Medical Research Council (B75-04-4-51-0) and the Research Council of the Swedish Sports Federation

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Reduction of the Tone of the Isolated Human Umbilical Artery by Indomethacin, Eicosa-5,8,11,14-Tetraynoic Acid and Polyphloretin Phosphate

By

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Received 23 December 1974

Abstract

STRANDBERG K and T TUVEMO *Reduction of the tone of the isolated human umbilical artery by indomethacin eicosa 5 8 11 14 tetraynoic acid and polyphloretin phosphate* Acta physiol scand 1975 *94* 319–326

The effects of 2 prostaglandin synthetase inhibitors indomethacin and eicosa 5 8 11 14 tetraynoic acid (ETA) and of the prostaglandin antagonist polyphloretin phosphate (PPP) on the tone of the isolated human umbilical artery and on the responses of this preparation to 5 hydroxytryptamine (5 HT) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were investigated Indomethacin (8 µg/ml) ETA (5 µg/ml) or PPP (40 µg/ml) reduced the tone of human umbilical arteries but had no influence on the responses to 5 HT In these concentrations ETA and PPP but not indomethacin antagonized the action of PGF_2 When the concentration of indomethacin or PPP was increased 5 fold both 5 HT and $PGF_{2\alpha}$ induced contractions were antagonized indicating a non-specific inhibition at these concentration levels A 10-fold increase in the concentration of ETA had no antagonizing action on 5 HT induced contractions suggesting a more selective inhibition of the PGF action than displayed by the other compounds The effects on the tone of the human umbilical artery of the compounds studied might reflect inhibition of prostaglandin biosynthesis and/or antagonism of the action of formed prostaglandins The findings are compatible with the view that intramural synthesis of prostaglandins contributes to the maintenance of the tone of the isolated human umbilical artery

It has been suggested that intramural synthesis of prostaglandins (PGs) maintains the resting tone of gastrointestinal smooth muscle This hypothesis was based on the findings that PGs are generated in isolated intestinal tissues and that concomitantly with the inhibition by indomethacin of this biosynthesis there is a reduction in tone (Ferreira Herman and Vane 1972 Eckenfels and Vane 1972)

Tuvemo and Wide (1973) found $PGF_{2\alpha}$ activity as measured by radioimmunoassay in the bath fluid of the isolated human umbilical artery Addition of indomethacin inhibited the appearance of PGs and the resting tone of the preparations was reduced An intramural

Part of this work was presented at the XIV International Congress of Pediatrics in Buenos Aires Oct 3 9 1974

synthesis of PGs in the human umbilical artery might have significance for the regulation of the umbilical blood flow as the PGs have been shown to exert both constrictor and dilator actions on this vessel (Altura *et al* 1972 Hillier and Karim 1968 Park Rishor and Dyer 1972)

To further analyse the possibility that PG formation contributes to the maintenance of the tone of the isolated human umbilical artery we have studied the effect on this preparation of 2 chemically unrelated PG synthetase inhibitors indomethacin (Vane 1971) and eicosa 5 8 11 14-tetraynoic acid (ETA) (Ahern and Downing 1970) and of polyphloretin phosphate (PPP) a polymer with PG antagonizing action (for references see Eakins and Sanner 197?) By also investigating the effects of the compounds on the contractile responses to $PGF_{2\alpha}$ and 5 hydroxytryptamine (5 HT) an evaluation of their specificity was obtained

Material and Methods

Preparation

Human umbilical cords from full term deliveries were taken immediately after cord clamping They were transported to the laboratory in Krebs-bicarbonate-glucose solution (KBG) as described by Furchgott (1960) From the fetal part (≤40 cm) of the cord spiral artery strips were carefully prepared (Furchgott 1960) They were transferred to 5 ml organ baths and mounted for isotonic recording using a Harvard smooth muscle transducer and a Servogor II recorder The load was 1 g The preparations were usually mounted within 10-20 min after delivery The organ baths contained KBG which was aerated with a gas mixture of 15 O_2 and 5 CO_2 in N_2 giving a pO_2 value of about 100 mm Hg and a pCO_2 value of about 40 mm Hg The temperature was kept at 37 C and the pH at 7 30-7 40 For this study 192 strips from 113 umbilical cords were used

Standardization

Standardization was carried out as follows

After an equilibration period of 60 min in the bath 5 HT (0 2 μg/ml) was given and 15 min later washed out Papaverine (100 μg/ml) was then added to the bath and left for 60 min Due to the long lasting nature of the complete experiments single doses of 5 HT and papaverine were used for the standardization The concentrations of the agonists were selected to give submaximal responses rather than supramaximal responses in order to retain reactivity for both constrictor and relaxing effects The tone level reached after 60 min exposure for papaverine was designated the zero level (0) and that after 15 min with 5 HT the standard response level (100) All results presented are expressed in percentages of the standard reponse

Effects on tone

The effect of indomethacin (Merck Sharp & Dohme) eicosa 5 8 11 14 tetraynoic acid (ETA) (Hoffmann La Roche Inc) or polyphloretin phosphate (PPP) (batch 101 k AB Leo Helsingborg Sweden) on the tone of the isolated human umbilical artery was studied as follows

After standardization cumulative dose-response relations for either 5 HT or PGF_2 were established After washing the onset of a steady decline or a steady state in tone was awaited Then indomethacin ETA or PPP was added to the bath The effect on tone of the drug was calculated as the difference between the level obtained 20 min after drug addition and the level expected at that time if the steady decline (or steady state) had continued

Specificity

For analysis of a possible inhibiting action of indomethacin ETA and PPP on the responses of the test preparation to agonists it was primarily decided to compare the dose response relations for the agonists in the presence and absence of the inhibitors However this approach was not feasible for the following reasons

Firstly the magnitude of the contractile responses to the agonists were found to vary significantly with the preexisting tone as illustrated in Fig 1 Hence it was concluded that dose-response curves could be compared only when they originated from the same level of tone Secondly preliminary experiments showed

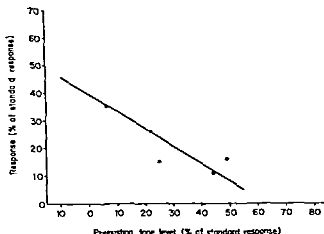


Fig. 1 Relation between the contractile response and the pre existing tone level. Single doses (2 μg/ml) of 5 HT were administered at different tone levels of the preparations. Washing followed on recording of each response. Results from experiments on seven preparations, denoted by different symbols, are presented. For clarification the calculated mean regression line is shown. For each experiment $r \geq 0.96$ $p < 0.001$

that many strips resumed a submaximal tone on washing out the papaverine after the standardization procedure. To compare dose–response curves starting from a high level of tone would not be appropriate since at this level large increases in concentration of an agonist are likely to produce only small increases in response. Thirdly it was discovered (see Results) that addition of an inhibitor to the bath reduced the tone of the test preparation, and more so at a high level. Thus introduction of an inhibitor to the test system might tend to antagonize the smooth muscle contracting action of any added agonist. To overcome these problems it was necessary to start the dose–response curves from a low level of tone and to compensate for the tone reducing effect of the inhibitors under study. For this purpose the control cumulative dose–response curve was produced immediately after the termination of the standardization procedure, *i.e.* at a low starting level of tone produced by retaining the papaverine in the bath. After the control dose–response relations had been established the strip was washed and an inhibitor was added to the bath. When the tone had decreased to the level at which the control dose–response curve started the second dose–response curve was produced. Thus of the two dose–response curves produced in each experiment, one was constructed in the presence of papaverine and the other in the presence of an inhibitor. The influence of retaining the papaverine (100 μg/ml) was estimated in 6 crossover experiments in which $PGF_{2\alpha}$ was used as the agonist (Fig. 2). Papaverine produced a parallel shift of the dose response curve to the right ($p < 0.05$).

Reproducibility

A contraction–relaxation cycle took 1 1/2–2 h. To assess the influence of the time factor on the responses to the agonists dose–response relations were established twice with an interval of 1 1/2–2 1/2 h for a total of 9 preparations. The dose–response curves did not differ significantly as tested by analysis of covariance of the differences in response.

Statistical analysis

Determination of regression coefficients and analysis of covariance were performed as described by Dixon and Massey (1957).

Results

Effects on arterial tone

Indomethacin ETA and PPP all reduced the tone of the arterial strips. This action is illustrated in Fig. 3. The lowest concentration that consistently produced relaxation was for indomethacin 8 μg/ml, ETA 5 μg/ml and for PPP 40 μg/ml. These concentrations of the

The statistical calculation (analysis of covariance) was based on the differences between the individual responses obtained in the presence and in the absence of papaverine.

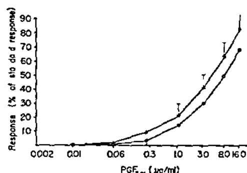


Fig 2

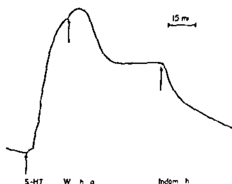


Fig 3

Fig 2 Effect of papaverine on the contractile response to $PGF_{2\alpha}$ In each experiment 2 cumulative dose-response curves to $PGF_{2\alpha}$ were determined one with papaverine (100 μg/ml) O—O and one without papaverine ★—★ using a cross over design Means and standard errors of the means for 6 expts are given

Fig. 3 Reducing effect of indomethacin (8 μg/ml) on the tone of the arterial strip The contractile response was produced by adding increasing amounts of 5 HT in total 3 μg/ml to the bath over a period of 18 min starting as ind cated by an arrow

inhibitors produced reduction in the tone by on the average 16% to 36% as calculated for a 20 min period (Table I) This effect seemed to be more marked when the pre-existing tone was high

Effects on 5 HT and PGF action

The responses to 5 HT were not inhibited by indomethacin 8 μg/ml ETA 5 μg/ml or PPP 40 μg/ml as compared to the responses produced in the presence of papaverine (Fig 4 a-c) On the contrary the responses were somewhat higher which most likely in part reflected the inhibitory effect of papaverine In an attempt to exclude the effect of papaverine from the analysis twelve experiments were carried out as follows The papaverine was washed out after the standardization and the first dose-response curve was constructed This was followed by washing and indomethacin (8 μg/ml) was added as before Then the second dose-response curve was produced In only 4 of the 12 expts the level of tone was comparable at the points where the 2 dose-response curves were started No significant difference between the responses to 5-HT with or without indomethacin in the bath was obtained

TABLE I Reducing effects of indomethacin (n = 16) ETA (n = 14) and PPP (n = 12) on the tone of the isolated human umbilical artery The results were calculated as the difference between the tone levels reached after 0 min and the levels expected if no inhibitor had been given The data for each inhibitor were subd ided into 2 groups of equal size as based on the initial tone of the individual experiments Means and ranges are presented

Compound	Tone reducing effect (of standard response)	
	low initial tone	h gh initial tone
Indomethacin (8 μg ml)	1 (7-46)	34 (1? 77)
ETA (5 μg ml)	23 (14-33)	36 (18-69)
PPP (40 μg/ml)	16 (11- 6)	? (12-45)

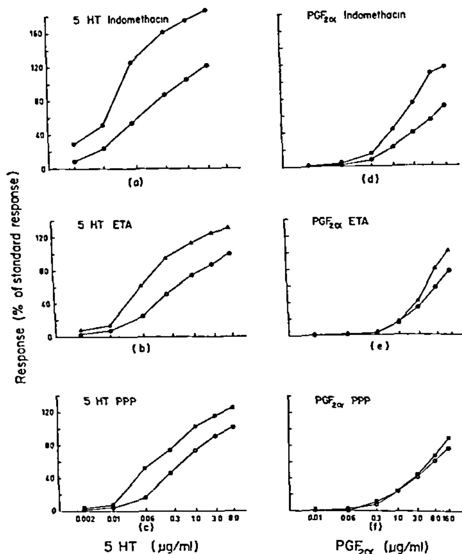


Fig 4 Cumulative dose response curves for 5 HT (a–c) and $PGF_{2\alpha}$ (d–f) in the presence of indomethacin (8 μg ml) ●—● ETA (5 μg/ml) ▲—▲ or PPP (40 μg/ml) ■—■ compared to controls (with papaverine 100 μg ml present) ○—○ Each curve rep esents the mean of 5–10 expts

The dose–response curves for $PGF_{2\alpha}$ in the presence or absence of inhibitor are presented in Fig 4 d–f As for 5 HT indomethacin (8 μg/ml) did not produce any inhibition of the $PGF_{2\alpha}$ induced responses In contrast the dose–response curves for $PGF_{2\alpha}$ were almost the same with or without ETA (5 μg/ml) or PPP (40 μg/ml) In view of the expected effect of papaverine corresponding to that seen in the experiments with 5 HT the latter results in dicated an antagonizing effect of these concentrations of ETA and PPP on the $PGF_{2\alpha}$ ac tion The inhibitory effects of the compounds now also including indomethacin were

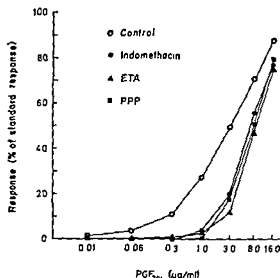


Fig 5 Cumulative dose-response curves for $PGF_{2\alpha}$ with indomethacin (40 µg/ml) ETA (50 µg/ml) or PPP (200 µg/ml) compared to control (with papaverine 100 µg/ml present) Each curve represents the mean of 4–5 expts

clearly demonstrated when the concentrations were increased 5 fold (indomethacin PPP) or 10-fold (ETA) (Fig 5) However at these concentrations indomethacin and PPP but not ETA antagonized the effects of 5 HT as well which indicated a non specific action

Discussion

Since the discovery by Vane (1971) that certain non steroidal anti inflammatory compounds possess PG synthetase inhibitory activity these compounds have been employed as probes to elucidate the physiological significance of the PGs (*cf* Vane 1973) The mechanism of action of the compounds in this respect is not known in detail but is regarded to be different to that of ETA (Lands *et al* 1973) As yet the PG antagonists available have been of limited value within this field of research They have either suffered from lack of specificity or have been active only in certain tissues and species (*cf* Eakins and Sanner 1972) However some of the PG synthetase inhibitors have effects in addition to that of suppressing the biosynthesis of the PGs Thus high concentrations of *e g* indomethacin and salicylates uncouple oxidative phosphorylation (Whitehouse 1964 Smith and Dawkins 1971) Moreover indomethacin has been reported to exert a membrane stabilizing effect possibly related to an action on Ca^{++} fluxes on guinea pig aortic and stomach smooth muscle (Northover 1971) The hazard in using the present PG synthetase inhibitors and PG antagonists for evaluations of the function of the PGs is thus obvious and the results must with necessity be interpreted with caution

The compounds used in this study were selected on the basis that they have different modes of action with respect to PG inhibition All of them *i e* indomethacin ETA and PPP were found to reduce the tone of the arterial strips in concentrations which did not antagonize the action of the non PG agonist 5 HT This was taken to indicate that none of the compounds acted via non specific depression of the activity of the smooth muscle cells

In addition ETA and PI P but not indomethacin exhibited an apparent antagonizing effect on the $PGF_{2\alpha}$ induced contractions Similarly Park and Dyer (1973) found an inhibitory effect of PPP (10 μg/ml) on PGE_2-induced contractions of the isolated human umbilical artery but no effect on the responses to 5-HT When in their experiments the concentration of PPP was raised to 100 μg/ml the responses to 5-HT were also antagonized indicating a low degree of selectivity for PPP This was confirmed in our experiments ETA was found more selective than PPP whereas the antagonizing action of indomethacin displayed in higher concentrations seemed to be non-selective

The finding of a reduced tone of the arterial strips on administration of the lower doses of either indomethacin ETA or PPP is compatible with the view that an intramural synthesis of prostaglandins controls the level of tone Indomethacin has previously been shown to abolish the production of $PGF_{2\alpha}$ activity in this preparation, as measured by radioimmunoassay (Tuvemo and Wide 1973) The two other compounds might also act via inhibition of the PG synthetase although an antagonism of the action of formed PGs cannot be excluded. Thus ETA has been shown to inhibit PG synthesis in the rabbit heart (Samuelsson and Wennmalm 1971) the cat spleen (Hedqvist, Stjärne and Wennmalm 1971) and to reduce the efflux of urinary PGE_2 in the rabbit (Larsson and Änggård 1974)

The mode of action of PPP in the present system is difficult to specify The compound was originally introduced as a high molecular weight enzyme inhibitor capable of inhibiting several enzymes *e g* alkaline phosphatase and hyaluronidase (Diczfalusy *et al* 1953) In addition to its PG antagonizing action, it has recently been found to possess inhibitory activities for both PG-dehydrogenase (Marazzi and Matschinsky 1972 Crutchley and Piper 1973) and PG synthetase (Perklev 1973)

If an intramural synthesis of PGs were of major importance for the maintenance of the tone of the umbilical artery also *in vivo* changes in this production would have impact on the blood flow Of special interest here is the closure of the artery that takes place at birth At birth the pO_2 of umbilical arterial blood is increased. The isolated perfused human umbilical artery has been reported to contract when the pO_2 is raised (Panigel 1962) Molecular oxygen is known to be needed for the biosynthesis of PGs (Samuelsson Granström and Hamberg 1967 Nugteren Beerthuis and Van Dorp 1967) The possibilities that an increase in the pO_2 stimulates the biosynthesis of PGs in the umbilical artery and/or promotes the contractile effects of formed PGs are presently under investigation

The skilful technical assistance given by Miss B Westerberg is gratefully acknowledged. We are grateful for the generous supplies of prostaglandins from Dr J E. Pike, UpJohn, and of polyphloretin phosphate from Dr B Högberg AB Leo

This investigation was supported by grants from the Medical Faculty University of Uppsala Sweden

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Acta physiol scand 1975 94 327–338

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Effect of Central Depressants on the Acoustic Middle Ear Reflex in Rabbit

A method for quantitative measurements of drug effect on the CNS

By

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Received 23 December 1974

Abstract

Borg E. and A R Moller *Effect of central depressants on the acoustic middle ear reflex in rabbit (A method for quantitative measurements of drug effect on the CNS)* Acta physiol scand 1975 *94* 327–338

The respective effects of pentobarbital–sod um enibumal–sodium urethane urethane–chloralose and lidocaine on the function of the acoustic middle ear reflex in the rabb t were studied The response of the middle ear muscles was measured by recording changes in both ears acoustic impedance when the reflex was elicited by applying pure tone stimuli (000 Hz) to the two ears one at a time In that way both the crossed and the uncrossed reflexes were stud ed All the drugs were found to depress the reflex in such a way that a higher sound intensity was required after administration to achieve the same impedance change as before The effect of the anesthetics was roughly proport onal to their known anesthetic power Lidoca ne produced only a sl ght depression of the reflex The crossed reflex showed a greater suscept b l ty to the general anesthetics than did the uncrossed reflex which suggests a greater complexity of the crossed reflex Because the method of record ng the reflex response does not require any surgery and is equally well applicable in unrestra ned rabbits and in humans it is suggested as a way of testing the effect of drugs on the central nervous system

The activity of the middle ear muscles (stapedius muscle and the tensor tympani muscle) can be measured in man and in the rabbit without any anesthesia or sedation Measurement of change in the acoustic impedance of the middle ear provides such a possibility and has been found to be both valid and reproducible in this light (Møller 1960 1961 1962 Borg 1972 a b)

A loud sound in one ear elicits a contraction of the middle ear muscles bilaterally though to somewhat lesser extent in the muscles in the contralateral ear (Moller 1961 1962, Borg 1972 b) In the rabbit the threshold of the ipsilateral reflex is about 70 dB re 2 10^{-4} N/m (SPL) for pure tones in the frequency range around 2.0 kHz. Both the stapedius and tensor tympani muscles of this species are activated by sound the threshold for the tensor tympani being about 10 dB higher than for the stapedius muscle In man only the stapedius muscle is activated as an acoustic reflex

The pathway of the acoustic middle ear reflex is in the lower brain stem The ipsilateral stapedius reflex arc contains 3 neurons Primary auditory neuron secondary neuron from ventral cochlear nucleus to the ipsilateral facial nucleus and the motoneuron The crossed stapedius reflex is activated through one additional relay in the region of the medial superior olivary nucleus There are in addition parallel multisynaptic connections (Borg 1973)

For further details on the functional properties of the middle ear reflex see e g Dallos (1973) and Møller (1972)

Although it has long been known that deep anesthesia abolishes the middle ear reflex only little quantitative information about the sensitivity of this reflex to anesthetics is available in the literature In a recent study in man (Borg and Møller 1967) a graded depression of the stapedius reflex excitability was obtained even at small dosages of pentobarbital sodium and ethylalcohol The reflex recordings in the study of Borg and Møller (1967) were obtained as changes in the acoustic impedance of the ear Since the same method is applicable to unanesthetized rabbits (Borg and Møller 1968) it was regarded favorable to analyze the effect of various anesthetic agents on the middle ear reflex system in rabbits and hence provide a broader material for purposes of comparison

In the present study the effect of some central depressants on the acoustic middle ear reflex in the rabbit was investigated The drugs were general anesthetics and a local anesthetic general modes of action of which differ Recordings of the sound-elicited activity of the middle ear muscles were taken immediately before and a certain time after intravenous administration of the drugs

Methods

periments were performed in a total of 27 rabbits most of which were used in several experiments with eral days in between The basis for the method used is that the acoustic impedance of the ear is a meas e f the mobility of the middle ear system A contraction of either or both the middle ear muscles changes he mobility of the middle ear system by increasing its stiffness gradually bringing forth a measurable change in the acoustic impedance The activity of the middle ear muscles can therefore be recorded by measuring the change in the ear s acoustic impedance which is largely proportional to the integrated EMG of the middle ear muscles (Borg 1972 a)

In the present study the change in acoustic impedance was measured in both ears simultaneously using the same method and set up as have been described earlier (Møller 1961 1963 Borg and Møller 1968) The two identical measuring devices each included three small hearing aid earphones one used for producing the measuring tone (800 Hz) the second used as microphone for recording the sound pressure of this tone in the ear canal and the third for delivering the sound stimulus to elicit the reflex (see Fig 1) The units were connected to the ear by a rubber tube sealed in the ear canal by a dental molding substance

The acoustic reflex was elicited by pure tones (2 000 Hz) delivered in bursts with a duration of 0 2 or 0 5 s The intensity was varied in steps of 2 or 4 dB Both the rise time to 90 of maximal amplitude and the decay time to 10 of maximal amplitude were ms The acoustic impedance change was measured in both ears simultaneously the stimuli were presented alternately to the left and right ear at the same time as the intensity was elevated stepwise from below threshold of the reflex to the maximal intensity employed The intensity was then lowered stepwise to below the threshold of the reflex In that way a given level of intensity appeared twice

The level of the stimulus sound at the eardrum depends on the anatomical properties of the individual ears which are likely to vary Therefore instead of relying on a standard calibration the stimulus sound pressure was measured in each experiment by using the earphone which usually delivered the 800 Hz measuring tone as a microphone This microphone including the narrow tube leading to the ear (see Fig 1) in turn was calibrated by means of a precision condenser microphone

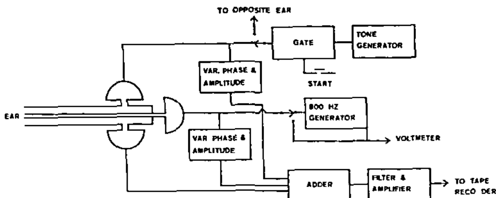


Fig 1 Block scheme of the apparatus for measuring the impedance change

The change in the ear s acoustic impedance caused by a middle ear muscle contraction was measured at a frequency of 800 Hz. The sound intensity of this tone was about 65 dB SPL which is well below the threshold of the acoustic middle ear reflex at 800 Hz. The output of the microphone was balanced out electrically in the absence of the stimulus signal (see Fig 1) When the middle ear muscles contract the ear s acoustic impedance changes and the balance is upset

The stimulus signal appears in the m crophone output circuit with an intensity which is far above the 800 Hz measuring tone Electrical filters are not sufficient to eliminate an interference of the stimulus tone if the dynamic propert es of the reflex are to be reproduced faithfully and therefore the stimulus signal was balanced out electrically in the microphone circuit in a similar way as was the 800 Hz measuring tone (see Fig 1)

The two 800 Hz signals which are a measure of the impedance change in the two ears together with a signal indi ating when the stimulus was on were recorded on magnetic tape by a two-track tape recorder and processed at a later occasion

The amplitude of the impedance change immediately before the end of the stimulus tone was measured and the values in percentage of the maximal obtainable value were plotted as a function of the stimulus level (in dB SPL) The mean of two determinations at each level was used to produce stimulus–response curves

During the experiments the rabbit was kept in a box open at the top and completely unrestrained The reflex recordings were performed before and at various time after admin stration of the drugs

Nembutal® Abbott (Mebumal sodium) was administrated intravenously dissol ed in Ringer solut on in concentrat on of 10 mg ml The doses used were 10 0 30 40 and 50% of the anesthetic dose (40 mg/kg) Narcotal® Astra 5 5 mg ml (Enibomal-sodium) urethane (00 mg ml) urethane–chloralose (100 mg/ml and 0 01 g/ml respectively) and Xylocain® Astra, . mg/ml (lidocaine) were administered by the same route A hyperdermic needle was cut and inserted in a vein in the pinna A plastic tube connected the cut end of the needle via a 2 way stopcock to two syringes one with the drug under investigation and one with Ringer solution The plastic tube was flushed with Ringer solution after each administrat on.

Results

Nembutal

22 rabbits (1 2–2 8 kg b wt) were used in a total number of 44 experimental sessions

Sensitivity of the reflex

The change in acoustic impedance was recorded immediately before and again 5 min after the Nembutal administration Fig. 2 illustrates a typical experiment in which the impedance change in both ears was recorded simultaneously when a pure tone of various intensities was presented to one ear Solid lines show ipsilateral and dashed lines contralateral response

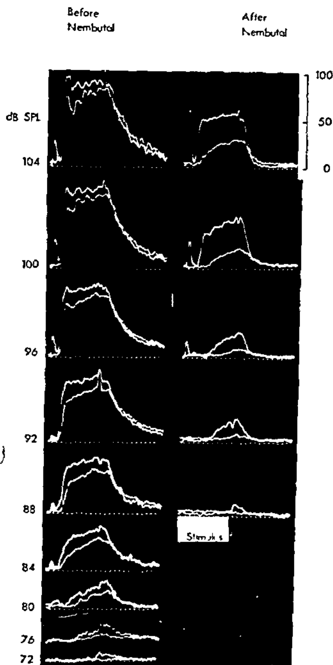


Fig 2 Change in acoustic impedance at various stimulus intensities as a function of time from before to 5 min after intravenous administration of 16 mg/kg Nembutal The solid lines represent the impedance change in the ipsilateral ear The stimulus was a 2 000 Hz tone with a duration of 200 ms The scale in the upper right corner gives the impedance change in percent of the maximal obtainable change

When the magnetic type recordings were played back the output of the two channels representing the impedance change of the two ears were aligned with respect to amplitude in such a way that the maximal impedance change obtained gave the same deflection for the two ears. The left column shows recordings immediately before administration of Nembutal and the right column recordings 5 min after administration of 16 mg/kg Nembutal

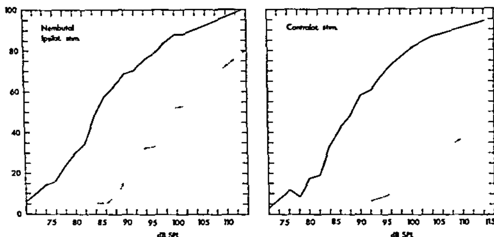


Fig. 3 Impedance change as a function of stimulus sound pressure level before (solid line) and 5 min after intravenous administration of 16 mg/kg pentobarbital. The response was elicited from the ipsilateral ear (left graph) and from the contralateral ear (right graph) with 200 ms long bursts of 2 000 Hz pure tones

The stimulus strength given in sound pressure level in dB SPL, measured near the eardrum, is shown to the left

It is seen in the left graph of Fig 2 that the magnitude of the response increases with increasing stimulus strength and that the contralateral response to the same stimulus strength is smaller than the ipsilateral The administration of Nembutal (right graph in Fig. 2) is followed by a decrease of the amplitude of the impedance change for the same stimulus strength and a rise of the threshold Furthermore the contralateral response is affected more than the ipsilateral response After Nembutal the time course of the response is changed in such a way that the onset is slower and the offset much faster The rapid offset probably signifies elimination of slowly conducting parallel pathways

Fig 3 shows the stimulus-response-curves of the ipsilateral and contralateral reflex before (solid lines) and after (dashed lines) administration of 16 mg/kg Nembutal The stimu

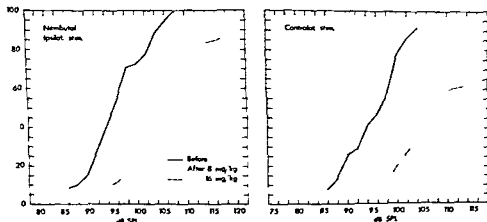
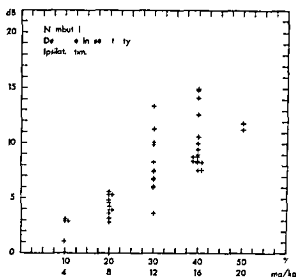


Fig. 4 Stimulus-response-curves before and after 8 mg/kg and 16 mg/kg of pentobarbital.



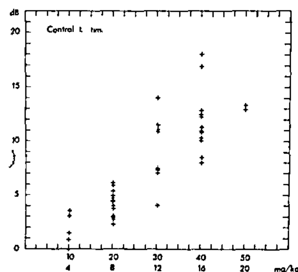


Fig 5 Increase in stimulus intensity necessary to obtain an unchanged response (20 impedance change) of the reflex to ipsilateral (upper graph) and contralateral (lower graph) stimulation at 2 000 Hz as a function of a dose of Nembutal

lus-response-curves are shifted to the right which implies that a higher sound intensity is needed in order to obtain the same impedance change as before Nembutal administration It is further observed that the sensitivity of the crossed reflex is affected more than that of the uncrossed reflex especially at higher sound levels The sensitivity is reduced in such a way as to get the same impedance change (20° of the maximally obtained) after Nembutal as before an elevation in sound level of about 13 dB being needed for ipsilateral stimulation and 20 dB for contralateral

Similar results from another experiment are seen in Fig 4 Two different doses of Nembutal were used here (20 and 40 of that required for surgical anesthesia) A dose of 8

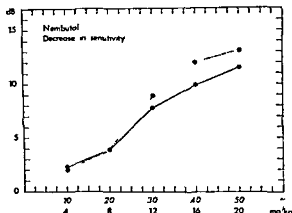


Fig. 6 The mean increase in stimulus intensity necessary to obtain an unchanged response (20% of maximal change) at 2 000 Hz as a function of a dose of Nembutal.

mg/kg reduced the sensitivity of the reflex by about 4 dB (measured at 20 % impedance change) Doubling the dose reduced the sensitivity by about 9 dB The difference between the sensitivity of the crossed and uncrossed reflex was not as pronounced in this experiment as in that illustrated in Fig 3

The effect of Nembutal on the sensitivity of the uncrossed (upper graph) and crossed (lower graph) reflex is shown as a function of dosage in mg/kg in Fig 5 The change in sensitivity is expressed as the number of dB which the stimulus tone has to be increased after Nembutal in order to elicit the same acoustic impedance change as before (20% of maximal obtainable) The results were obtained in 25 expts on 11 rabbits Each point in Fig. 5 represents one ear while Fig 6 shows the averages of these values It is evident from these two figures that Nembutal produces a measurable decrease in the sensitivity of the reflex at doses above 4 mg/kg. Furthermore a more pronounced effect on the crossed reflex than on the uncrossed one is seen for doses above 12 mg/kg ($p<0.05$ at 16 mg/kg $p<0.01$ t test) A potentiating effect of Nembutal on the reflex excitability has not been observed

Time course of the Nembutal effect

The time course of the reflex depression was studied by stimulation with repetitive tone bursts presented at a rate of 1 per 3 s A constant level of depression was reached some 20

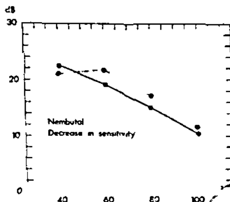


Fig 7 Increase in stimulus sound pressure of the uncrossed (solid line) and the crossed (dashed line) reflex necessary to obtain an unchanged response (20% of maximal) after intravenous administration of 40 mg/kg Nembutal.

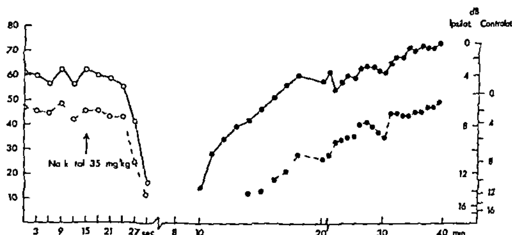


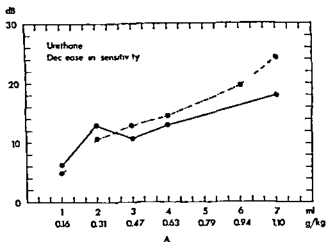
Fig. 8 Impedance change elicited by 200 ms long bursts of a 2 000 Hz tone in percent of maximal obtainable impedance change as a function of time after intravenous administration of 35 mg/kg Narcotal To the left impedance change and to the right decrease in excitability of the reflex in dB are depicted The open circles show the response to a tone with an intensity of 86 dB SPL in the left ear of the uncrossed (solid line) and crossed (dashed line) reflex The filled circles show the response to 92 dB tone

s after the injection of a subanesthetic dose of Nembutal The effect lasted at least 5-10 min *i e* during the routinely performed sensitivity test The time course of the reflex depression after a single anesthetic dose (40 mg/kg) of Nembutal is shown in Fig 7 The decrease in excitability was determined from the shift in the stimulus response-curves at 20° of maximal impedance change No reflex response was present at stimulus intensities below 114 dB SPL until 40 min after the injection of the anesthetic After 100 min the decrease in sensitivity of the reflex is the same as that seen 5 min after administration of 16–20 mg Nembutal (*cf* Fig 6)

The recovery of the reflex response after a single anesthetic dose of Nembutal was studied in 8 rabbits submitted to middle ear surgery The loss of sensitivity remaining after about 2 hours was 23 dB for the crossed response and 22 dB for the ipsilateral response

Narcotal

The effect of a single dose (35 mg/kg which gives surgical anesthesia) of Narcotal as a function of time was studied in 6 expts on 3 rabbits Fig 8 shows the depression and the restitution of the reflex activity in a typical experiment The left part of the figure shows the response (impedance change in per cent of maximal obtained change) to repetitive stimulation with 2 000 Hz tone bursts with an intensity of 86 dB SPL presented to the left ear The results shown in the right graph were obtained in a similar way with a sound intensity of 92 dB SPL applied alternately to the left and right The drug was injected intravenously during 50 s The beginning of the injection marked by an arrow was followed after about 10 s by a depressive action About 25 s after the beginning of the injection the stimulus strength used did not evoke any measurable response When the stimulus strength was increased to 92 dB SPL (right part of figure) the ipsilateral reflex reappeared after 10 min and the contralateral after 14 minutes Complete recovery took place after 30–40 min A



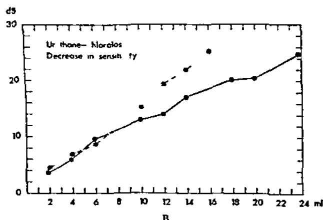


Fig. 9 Increase in st mulus sound pressure necessary to obtain 20 ⁿ impedance change as a function of a dose of urethan (upper graph) and urethane–chloralose (lower graph) Ipsilateral reflex represent ed by solid line and contralateral reflex by dashed line Stimulus 2 000 Hz pure tones of 00 msec duration *One ml urethane–chlor* alose contains 100 mg urethane and 10 mg chloralose

difference in sensitivity of the ipsilateral and contralateral reflex is seen also after reappear ance of the reflex activity The crossed reflex is about 4 dB less sensitive to stimulation than the uncrossed for as long as about 25 min aft r the induction of the anesthesia

Urethane urethane–chloralose

The effect of these drugs was studied in 1 expt each with cumulative doses The susceptibility of the reflex was determined from the shift in the stimulus–response-curve at each dose level Fig. 9 shows the effect of urethane (upp r graph) and urethane–chloralose (lower graph) on the uncrossed (solid line) and crossed (dashed line) reflex expressed as the increase in stimulus intensity necessary to obtain a certain impedance change As is seen the crossed reflex is more susceptible to both drugs especially at higher doses The addition of chloralose does not seem to decrease the reflex-depressant action of urethane

Xylocain

The effect of intravenously administered Xylocain was studied in 3 expts in 2 rabbits Fi 10 shows the stimulus-response-curves of the ipsilateral and contralateral reflex before

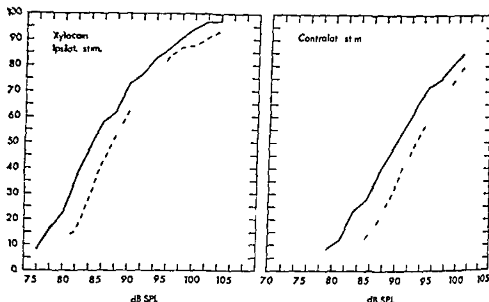


Fig 10. Stimulus-response-curves before (solid line) and after (dashed line) 10 mg/kg Xylocain i v

line) and after (dashed line) 10 mg/kg Xylocain The curve is shifted to the right equally much for both the crossed and uncrossed reflexes and the excitability is thus decreased about 3 dB.

Discussion

The results of the present study show that all of the general anesthetics investigated produced a dose dependent depression of the middle ear reflex No facilitation was ever observed The crossed reflex was more sensitive than the uncrossed to all but the smallest doses used The local anesthetic and antiepileptic Xylocain gave only a slight depression equal for both the uncrossed and the crossed middle ear reflexes

Several studies have shown that barbiturates in the doses used in these experiments have no depressant action on striated muscle contractions (Kraatz *et al* 1953 Borgman *et al* 1960) Nor are the cochlear potentials changed by anesthetics (see *e g* Lawrence 1960) It is thus possible to ascribe the observed depression of the reflex to an effect on central pathways

Studies by Arduini and Arduini (1954) and Nakai *et al* (1965 1966) have shown that the amplitudes of click evoked [illegible] tials both in the brain-stem reticular formation and in the ascending acoustic pathw[illegible] nished by gene[illegible] sthetics It is however uncertain what is due to action on [illegible] tic pathway pr[illegible] nd what is due to changes in the facilitation inhibition bia[illegible] er parts of [illegible] Multiple unit recordings made at various locations in th[illegible] thway (H[illegible] 1969) showed that background activity [illegible] depressed w[illegible] oked activ[illegible] er level of the auditory system was lar[illegible] hanged [illegible] rate anesth[illegible] marked depression of the [illegible] the ret[illegible] on The [illegible] auditory inflow for the a[illegible] dle ear [illegible] in the [illegible] by Hugelin *et al*

(1960) and Carmel and Starr (1963) Baust and Berlucchi (1964) found definite changes in the EMG pattern in the tensor tympani reflex after ablation of the acoustic cortex and similar changes were observed by Salomon (1966) after intercollicular decerebration In the rabbit (Borg 1973) a slow and Nembutal sensitive crossed stapedius reflex response remained after large lesions in the midline of the trapezoid body This is most likely generated by multisynaptic diffuse connections possibly via the reticular formation

As pointed out in earlier studies (Møller 1961 Borg and Møller 1967) there are some functional differences between the crossed and uncrossed middle ear reflex foremost the greater sensitivity of the ipsilateral to acoustic stimuli The difference in excitability between the ipsilateral and contralateral reflex expresses properties of the parts of the reflex arc that are unique to the contralateral reflex (Møller 1961) The difference between the two reflex arcs is mainly the presence of a direct connection from the ventral cochlear nucleus to the ipsilateral 7th motor nucleus and the absence of any such connection with regard to the contralateral facial motor nucleus The greater sensitivity of the contralateral reflex to general anesthetics as shown here and by Borg and Møller (1967) answers to the expectations to which these reflex arc differences give rise

The difference in drug sensitivity between the crossed and uncrossed reflexes derives from the properties unique to the crossed reflex From Fig 6 it is seen that at a dose of 16 mg/kg the increase in stimulus intensity necessary to obtain an undiminished impedance change is 4 dB greater for the contralateral reflex than for the ipsilateral

Intravenously administered Xylocain in doses several times those sufficient to block epileptic seizures had only very slight depressive action on the middle ear reflex This is in accordance with its demonstrated low effect on other polysynaptic reflexes (Blom 1963)

As seen from Fig 6 and 9 fifty per cent of the dose for surgical anesthesia of Nembutal urethane and urethane–chloralose gives almost equal depression of the contralateral reflex while Xylocain has in principle no effect on the middle ear reflex or on consciousness The sensitivity of the middle ear reflex to the drugs studied thus seems to be related to their effect on consciousness *i e* probably due to an effect on the reticular formation By comparison the middle ear reflex in humans (Borg and Møller 1967) is much more sensitive to Nembutal than that in the rabbit taking body weight into account This finding agrees with the proportions generally assumed *i e* about 5 times smaller anesthetic dose for human than for rabbits

It is suggested that measurement of the middle ear muscle reflex excitability can be useful for the determination of drug effects Both the degree and the time course of the effects can be studied conveniently

This work was supported by Therese and Johan Anderssons Minne Folksams Forskningsfond and the Swedish Medical Research Council (project 04X 90 and P 3 51)

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The occurrence of 2 intermingled sets of capillaries running in parallel and with very little interstitial tissue inbetween them makes the *rete* especially suited for studies of capillary permeability Such investigations have been performed (Stray Pedersen and Steen 1975 Stray Pedersen 1975) In order to analyze and to understand the data obtained in such studies one needs however structural information on the capillaries under study It is generally assumed that the permeability characteristics of different types o capillary walls depend on their specific structure which may vary from species to species and especially from one organ to another within the same species (Bennett *et al* 1959) For the computation of permeability and diffusional constants in the *rete* one will thus need exact informations about the actual size of important structural parameters such as the surface and the thickness of the retial capillary barrier Ultrastructural studies of the retial vessels have been performed by Dorn (1961) and by Fawcett (1963) in organs fixated by immersion and recently by Bendayan *et al* (1974) in organs fixated by perfusion Neither of these studies give however sufficient quantitative information for a satisfactory functional analysis

In the present study qualitative and quantitative morphological studies have been carried out To this end light microscopical as well as electron microscopical examinations have been performed The ultrastructural studies were predominantly carried out in preparations which were fixated through perfusion since this method appears to give a more sudden arrest of the tissue situation than immersion fixation

The ionic composition of the endothelial cells were evaluated by measuring the concentrations of sodium potassium calcium and magnesium in retial tissue and in eel erythrocytes

Materials and Methods

Gross vascular anatomy As seen in Fig 1 the swimbladder of the eel has two *retia* which get their blood supply from a branch of the swimbladder artery the *pre rete* artery At the distal pole of the *rete* the arterial capillaries reunit into two or more *post rete* arteries which again divide and give origin to the capillary network of the secretory bladder The venous blood is presented to the *rete* at its distal pole in the *p e rete* veins which split into numerous venous capillaries running parallel to the arterial ones but in the opposite direction At the proximal pole of the *rete* the venous capillaries reunit into a single *post ete* vein Thus the blood to and from the secretory bladder has to pass through a complex vasculature consisting of 3 successive capillary systems or portal systems of which the first and the last ones are situated in the site

Retia from eels of different sizes were employed in this investigation The animals which almost exclusively represented silver eels were completely adapted to fresh water The eel was either made unconscious by a blow on its neck or else anaesthetized by submersion in a 0 05 solution of MS-222 for 5 min *The eel was fastened in an eel holder (Steen 1963 a) and the gills irrigated with tapwater* The abdomen was then opened and the *p e ete* artery and the *post rete* vein cannulated The vessels distal to the *rete* were cut and the *ete* perfused through the two cannulated vessels

Light microscopical studies The *r t a* were dissected out, fixated in 10 formaline and stained with hematoxylin-eosine (H-E) Cross-sections as well as longitudinal sect ons of the *retia* were examined

The quantitative measurements were performed on cross sectioned *retia* fixated in 10% formal ne for 4 -7. h (section thickness 10 μm stained with H E) Since it was easier to distinguish between arterial and venous capillaries when the blood cells had been washed out a short lasting perfusion with plasma or with Ringer solutions containing papaverine in order to paralyze the vasculature (Stray Pedersen 1970) was performed prior to the fixation The shrinkage due to the fixation was evaluated by comparing the circumferences of the *retia in situ* with those of the correspond ng preparations after fixation Th s latter type of circumference was measured on the 10 μ thick cross-sections which were magnif ed by a slide projector Measurements on *10 retia of very different sizes showed that corrections* of about 10% and 20% were necessary for the length and the area values respecti ely in the fixed preparations

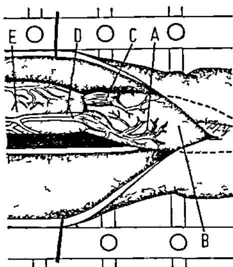


Fig 1 A drawing of the swimbladder of an eel intestines and gonads removed. A = *Post rete* artery and *pre rete* vein B = secretory bladder C = *rete* D = *pre rete* artery and *post rete* vein E = reabsorbent bladder

The total cross sect onal areas of the *etia* were determined very accurately by putting the microscopical sections into a slide projector and then drawing the outlines of the depicted preparations on mm paper The actual magnif cat on was determined by means of a micrometer arrangement in the projector

The number of arterial and venous capillaries was counted through a microscope equipped with an ocular grid The count ngs were performed in at least ₋0 small squares with a side length of 0 0075 cm, each square contain ng on an average 25 capillaries In one preparation (preparation E in Table I) the number of capillaries as well as the cross sectional area was also estimated on microphotographs The countings were performed in superficial as well as in central parts of the *rete*

From the values obtained for the total number of capillaries (N_T) and the cross-sectional area (A_C) the mean capillary rad us (r) in the *rete* could be computed accord ng to the formula

$$r = \sqrt{\frac{2/3\ A_C}{\pi\ N_T}} \tag{1}$$

assuming the area constituted by tissue to be 1/3 of the total cross-sectional area (Krogh 1929) In two preparations the diameters of the cap llaries were also measured directly with an ocular m crometer

The total surface of each cap llary system (A) in the *rete* was calculated according to the formula

$$A = \tfrac{1}{2} N_T\ 2\ \pi\ r\ L \tag{2}$$

in which L represents the capillary length e assuming $N_A \approx N_V$

Elect on m c oscop cal studies were performed predominantly on perfusion f xated but also on immersion fixated cross sectioned *ret a* Prior to the fixat on the *rete* was perfused with a Ringer solution containing 3 Dextran (Dextran T 80 Pharmacia) The fixation was performed by perfusion of a solution conta n ng 1 7 glutaraldehyde in 0 08 M phosphate buffer (pH 7 4) for 15–20 min The *te* de eloped a uniform yellow tinge after few min Samples of the retial tissue were then immersed for 2 h in ice-cold 3 glutaraldehyde in 0 08 M phosphate buffer then cut into small blocks of about 1 mm and washed overnight in ice-cold Tyrode buffer The blocks were post f xated for 1 h and 30 mi n ce-cold 1 OsO_4 in Tyrode buffer (pH 7 4) washed briefly in Tyrode buffer dehydrated in graded al ohols and embedded in Epon 812 Semithin sections were stained with toluidine blue and examin d by light microscopy Ultrathin sect ons were cut with a LKB-Ultratome ultra m crotome sta ned w th ur yl acetate and lead citrate (Reynolds 1963) and examined in a S emens Elmiskop I electron m croscope

M a ements of elect olytes i retial t ss e and e th y The *ete* was perfused with plasma or Ringer solutions in order to wash out the blood and n some experiments inflat d with air The cap llary di ision of the organ was d ssected out cut into slices each weighing 10– 0 mg, wh ch were carefully

TABLE I Cross-sectional areas number radii and surface areas of capillaries in 5 different *retia*

Preparation	Cross-sectional area of the *rete* (A_C) (mm²)	Number of arterial capillaries (N_A) ± S D	Number of venous capillaries (N_V) ± S D	Total number of capillaries (N_T)	Calculated mean capillary radius (r) (μ) $\bar{r} = \sqrt{\tfrac{1}{3}A_C/\pi N_T}$	Measured mean capillary radius ($\bar{r}$) (μ)	Calculated capillary surface area (cm² A) $A = \tfrac{1}{2} N_T 2 \pi r l$
A	20 56			182 500	4 89		112
B	5 25	33 600 ±4 800	21 500 ±3 700	55 100	4 50		31
C	3 88	22 100 ±3 500	15 200 ±1 500	37 300	4 70	5 33	[illegible]0
D	10 70	51 300 ±4 200	38 000 ±3 100	89 300	5 05	4 82	57
E	9 15	55 400 ±6 300	34 400 ±5 400	89 800	4 65		52
Krogh's *rete* (Krogh 1929)	8 0	58 000	44 000	102 000	4 08	4 2	52

squeezed on filter paper in order to remove as much intravascular fluid as possible The tissue slices were dissolved into 200 μl of hot concentrated sulphuric acid 200 μl of H_2O was added and the concentrations of the various ions were measured in this solution The fact that the various ion concentrations in these solutions were found to be identical with the concentrations measured in the supernatants obtained after centrifugation of this solution indicated that the tissue had become completely disintegrated during the treatment with sulphuric acid The concentrations of potassium and sodium were determined by flame [illegible]ry using an Eppendorf flame photometer The concentrations of calcium and magnesium were measured with an Unicam atomic absorption photometer Blank analyzes with the solution of concentrated sulphuric acid and water were performed in order to correct for the contamination of sodium from the glass

In order to compare the values obtained from retial tissue with those from other homologous cells, similar analyses were performed on eel erythrocytes The red cell suspension was obtained by centrifuga

TABLE II The ionic composition of the retial tissue and of eel erythrocytes as compared to that of human erythrocytes

	Eel *rete* mM/l	Eel erythrocytes mM/l	Human erythrocytes mM/l
K^+	61 ± 13 (S D) n = [illegible]4	84 n = 2	136 [1]
Na^+	73 ± 25 (S D) n = 4	[illegible]3 n = [illegible]	19 [1]
Ca^+	4 n = 2	5 n = 1	0 0[illegible] [2]
Mg^{++}	9 n = 2	12 n = 1	3 3 [2]

References [1] Bernstein (1954) [[illegible]] Harrison and Long (1968)

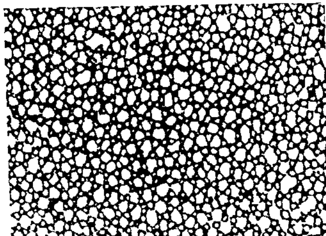


Fig 2 Micrograph of a cross sectioned *rete* (×450) which has been perfused with horse plasma prior to the fixation The arterial and venous capillaries may easily be identified the arterial vessels having smaller diameters and thicker walls The venous capillaries, however are considerably distended in this preparation as a result of the conditions prevailing during the perfusion.

tion of eel blood at 1000 g for 20 min The values were corrected for the inevitable contamination of the cell sediment with plasma (4%)

The accuracy of getting ionic composition from biological specimens heated with sulphuric acid was tested by carrying out the same procedure on plasma The values obtained with this method were seen to be accurate to within ±10 when compared to the values obtained by direct ionic measurements in plasma The reliability of the method for measurement of intracellular ion concentrations was evaluated by carrying out similar analyzes of the ionic content in human erythrocytes The values obtained were in the ranges of 144 169 meq/l for K^+ and 12-28 meq/l for Na^+ and thus not essentially different from the values reported by other investigators (Table II)

Results

Light microscopical studies

On cross-sectioned preparations the very regular arrangement of the retial capillaries could be recognized (Fig 2) The arterial and venous capillaries could easily be distinguished in the preparations in which the red cells had been washed out the arterial capillaries having smaller lumina and thicker walls An arterial capillary was surrounded by 3 to 7 (in most cases 4) venous ones and *vice versa* The capillaries located close to the surface did often have a somewhat smaller calibre than the central capillaries However the opposite phenomenon was also observed in some preparations Structures resembling arterio-venous anastomoses were never observed Nor were discontinuities in the capillary walls ever seen

On preparations sectioned longitudinally the capillaries were seen to be straight or slightly curved At both ends of the capillaries numerous melanophores could be detected

Quantitative estimations Macroscopically the capillary division of the *rete* was distinctly marked by a narrow pigmented zone at both poles due to the presence of numerous melanophores in these areas The capillary length could therefore be measured directly In 10 *retia* of very different sizes an almost constant length of 4 mm was obtained

Measurements of the cross-sectional area and of the number of capillaries were performed on 5 *retia* of very different sizes In one preparation (preparation A in Table I) it was impossible to distinguish between the arterial and venous capillaries and hence only the total

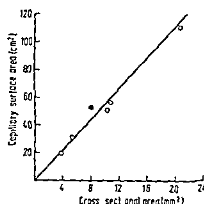


Fig 3 The relationship between the measured values for cross sectional area and the calculated values for total capillary surface area in 5 *retia* (see Table I) (The black circle represents values given by Krogh for the *rete* examined by him (Krogh 1929)

number of capillaries could be estimated In the remaining four *retia* the arterial and venous capillaries were counted separately The number of capillaries arrived at as well as the mean capillary radii and surface areas calculated according to Eqs 1 and 2 are listed in Table I and compared to the values obtained by Krogh (1929) Fig 3 shows the relationship between the capillary surface area and the corresponding cross sectional area in these *retia*

In two preparations (preparations C and D in Table I) the capillary diameters were measured directly (n = 100) The diameters were found to be as follows (mean values ± S D) d_A = 8 64 μm ± 1 43 and d_V = 13 62 μm ± 1 87 in preparation C and d_A = 8 70 μm ± 1 25 and d_V = 10 90 μm ± 2 08 in preparation D giving mean capillary radii of 5 33 μm and 4 82 μm respectively (calculated according to the formula $r = (N_A r_A + N_V r_V)/N_T$) total capillary surface in the arterial and venous system in these preparations would be A_A = 24 cm² and A_V = 26 cm² in preparation C and A_A = 56 cm² and A_V = 52 cm² in preparation D As seen from Table I these values are not essentially different from those computed from the values of the capillary number and the cross sectional area assuming the retial tissue to represent 1/3 of the cross sectional area (Eqs 1 and 2)

Ultrastructural studies

Even at low magnification the arterial and venous capillaries were easily identified (Fig 4) On cross-sections the arterial capillaries were approximately circular in outline and had rather thick walls The venous capillaries were more irregular in outline and had considerably thinner walls In both types of capillaries the main elements of the general structure of capillary walls could be recognized (Fig 4) The endothelium and the basement membrane were surrounded by a layer of interstitial tissue (adventitia) In each complete cross sectional cut of endothelium parts of some 4–7 endothelial cells could be recognized In the spaces between the cell the junctions could be observed as darker regions The endothelial cells contained numerous vesicles and vacuoles thus giving the cytoplasm a spongy appearance Each capillary was surrounded by a distinct basement membrane whereas the adventitia appeared as a less well defined layer especially in those regions in which the arterial and venous capillaries were in close contact with each other By far the greatest

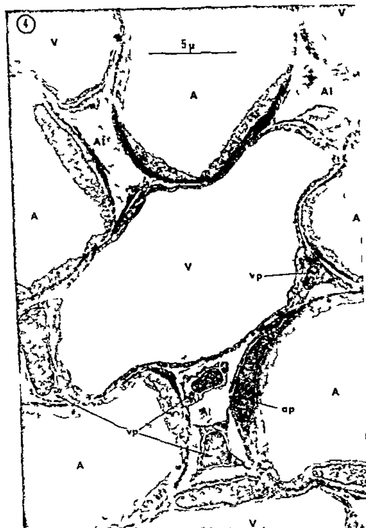


Fig 4 Electron micrograph of a cross sectioned perfusion fixated *rete* showing a venous capillary (V) in center surrounded by 5 arterial capillaries (A) Between the fragments of the endothelial cells the intercellular junctions may be recognized as darker regions In the angular interstice (Al) at the bottom of the picture a venous pericyte (vp) is seen to be located freely in the interstice whereas the arterial pericyte (ap) appears to be embedded in the basement membrane of the arterial capillary

fraction of interstitial tissue was confined to the angular interstices which also contained the perikarya of the pericytes (adventitial cells)

Since the endothelia of the arterial and venous capillaries were essentially different in structure they should be described separately

Arterial endothelium The endothelium had its greatest thickness in the nuclear regions (about 1 μm) and was more or less attenuated towards the periphery the average thickness being about 0 3 μm At the levels of the intercellular clefts the height of the endo

was often seen to be markedly diminished in such a fashion as to make the appearance of cavities in these regions

The plasmalemma had a thickness of about 100 Å and did not seem to differ in structure from the usual unit membrane The structure was uniform along the entire perimeter of the cell and was not affected by local differentiations such as plasmalemmal invaginations or cell protrusions

The cytoplasm contained the usual complement of cell organs The Golgi complex, the endoplasmatic reticulum the ribosomes and the mitochondria were generally concentrated in the nuclear region The ribosomes were predominantly confined to the endoplasmatic reticulum, free ribosomes were rather infrequently encountered. The mitochondria were rare but of prominent sizes

The cytoplasma was crowded with pinocytotic (plasmalemmal) vesicles (*e g* Fig. 8-11) The population of the vesicles was greatest in the attenuated parts of the endothelium The vesicles were circular or ovoid in outline with diameters between 200 and 800 Å and were bounded by a membrane similar to the plasmalemma. They were especially numerous along the two borders of the cell in the immediate vicinity of the cell membrane thus indicating that they represent plasmalemmal invaginations About 1/3 of the vesicles were located freely in the cytoplasm Many vesicles were seen to open onto the luminal or subendothelial space (Fig. 9) or onto the intercellular clefts (Fig 6) either directly or through a narrow neck. Some vesicles were separated from these spaces by a diaphragm which was continuous with the cell membrane but considerably thinner than this (about 50 Å) (Fig. 9) others were attached to the surface by strands of plasmalemma. The vesicles contained various amounts of fine granulated material or appeared to be empty No systematic variations of the vesicles, especially with respect to their sizes and content could be observed along the luminal abluminal axis In some places chains of 2–4 intercommunicating vesicles were observed, some of which were seen to open at the cell surface However a continuous chain of intercommunicating vesicles from the luminal or the intercellular space to the subendothelial space was never observed

The cytoplasm also contained vacuoles of considerably greater sizes (*e g* Fig 4 and 5) These vacuoles were more or less elongated with a width between 0 1–0 3 μm, and a length between 0 1–1 5 μm Similar to the pinocytotic vesicles they were most frequently located in the peripheral parts of the cell The content of these large vacuoles appeared to be similar to that observed in the lumen (Fig 14)

The nuclei of the endothelial cells were seen to be indented and often bent to follow the curvature of the vessels (Fig. 4) The nuclear plasma had a fibrillar or granular structure and contained irregular masses of chromatin

At the levels of the intercellular clefts the two adjacent endothelial cells would show different degrees of overlapping. The length of the clefts could therefore vary considerably Some had the appearance of short, straight running channels with a length of about 500 Å (Fig. 5 and 9) whereas other clefts appeared as tortuous slits (Fig. 6) having a length of several μ The diameter of the clefts varied between 100–250 Å. The clefts appeared to have a random distribution, i.e they were located just as frequently in the endothelium facing the angular interstices as in other regions of the wall In some places, however a juxtaposition

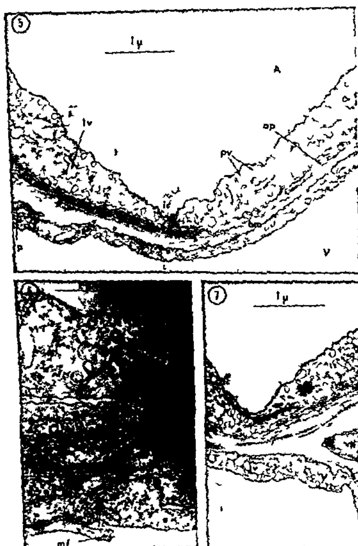


Fig. 5 Electron micrograph of a cross sectioned perfusion fixated *rete* showing the arterio-venous barrier at a site of minimal barrier The content of pinocytotic vesicles (pv) is seen to be significantly higher in the arterial than in the venous endothelium In both endothelia large vacuoles (lv) are observed In the luminal half of the intercellular cleft (ic) of the arterial capillary a desmosome is recognized

Fig. 6. Electron micrograph of a cross-sectioned perfusion fixated *rete* showing an arterial and a venous intercellular cleft (ic) in a juxtaposition In the arterial cleft, which appear to have a patent gap junctional structures seem to be absent A narrowing of the space between the cell membranes may however be observed at a distance of 0.5 µm from the luminal surface Pinocytotic vesicles partly intercommunicating open onto the cleft. In the region close to the venous intercellular cleft a marginal fold (mf) is observed

Fig. 7 Electron micrograph of a cross-sectioned perfusion fixated *rete* showing an arterial intercellular cleft (ic) with a more centrally located desmosome At both ends of the desmosome narrowings of the intercellular space are recognized In these regions (not so distinct in this picture) an increased accumulation of dense material partly obliterating the intercellular space These structures most probably represent special attachment devices (see text)

of an arterial and a venous cleft as shown in Fig 6 and 10 was observed In certain regions of many clefts usually in their luminal thirds elements of denser junctional structures with the appearance of desmosomes could be observed (Fig 5 and 7) Usually the junctional spaces seemed to contain masses of material with light density In all the clefts including those where no desmosomes could be seen one could observe a narrow region extending for 100–300 Å The average diameter of the junctional gaps in these regions was found to be about 110 Å as measured on 20 junctions

Venous endothelium The endothelium varied considerably in thickness but it was generally found to be thinner than in the arterial capillaries (about 0 15 μm)

In some places (Fig. 9 12 and 13) smaller regions of discontinuities of the cell wall could be observed These regions the so-called fenestrae appeared to be similar to those described in some mammalian capillaries (Rhodin 1962 Luft 1965 Maul 1971) The diameters of the fenestrae were between 200–800 Å Each fenestra was provided with a diaphragm (of about 50 Å in thickness?) which was continuous with the plasmalemma (Fig. 9) A central knob in the diaphragm could not be detected The fenestrae were most frequently encountered in the attenuated parts of the endothelium especially in those regions in which the total arterio-venous barrier was very thin Fenestration of the venous capillary walls facing the angular interstices was in fact very uncommon The number of fenestrae in the venous capillary walls seemed to be much smaller than what has been observed in mammalian visceral capillaries (Friederici 1968 Clementi and Palade 1969) On many of the present venous capillary profiles fenestrae were completely absent When observed however they were usually arranged in clusters of 3–5

The cytoplasm and the nucleus of venous capillaries had a less dense structure than in arterial capillaries but the same structural elements were encountered in both types of capillaries The concentration of pinocytotic vesicles was significantly smaller in venous capillaries though At the luminal surface which appeared to be more irregular than in the arterial endothelium clusters of microvillous processes could be observed In many instances as shown in Fig. 6 12 and 14 such cytoplasmic extrusions could be recognized near the intercellular clefts (marginal folds) and in the close vicinity of one or more giant vacuoles

The intercellular clefts (Fig. 6 9 and 11) seemed to be similar to those in the arterial capillaries In the venous capillaries they appeared to be more straight running and somewhat wider though having diameters between 100 and 300 Å In their narrow regions the diameters were about 120 Å

The basement membrane had a thickness of 100–150 mμ and contained numerous microfibrils On the majority of the micrographs (not so distinct on those shown in the figures) a three layered structure of the membrane such as described by Dorn (1961) could be more or less recognized In most places the arterial and venous basement membranes appeared as separate distinct structures However in those regions in which the arterial and venous capillary walls were in intimate contact the basement membranes were seen to be more or less fused In some instances it was difficult to distinguish between the basement membrane proper and the underlying interstitial tissue in that both elements made up one more or less continuous layer (Fig 12 14 Note that the black spots observed in these figures represent accumulations of horse radish peroxidase molecules) It is possible that this layer

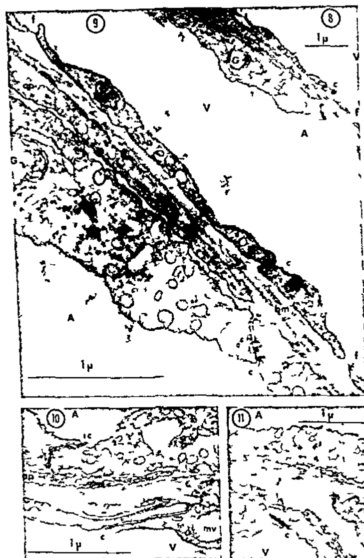


Fig. 8 Electron micrograph of a cross-sectioned perfusion f xated *rete* showing the arterio enous barrier in a region in which the venous capillary wall shows fenestrations. The venous endothel um is thin and prov ded with 2, perhaps 3 fenestrae (f) s tuated close to the intercellular cleft. In the arterial capillary a Golgi apparatus (G) is observed

Fig. 9 Same as in Fig. 8 with a higher magnif cation showing details in the structure of the fenestrae (f) The diaphragms of the fenestrae are significantly thinner than the plasmalemma (about 50 Å) The intercellular cleft (c) is ery short (about 500 Å) and to contain denser material

Fig. 10 Electron micrograph of a cross-sectio ed perfusion-f xated *rete* showing a venous intercell lar cleft (ic) having a continuous space A marginal acuole (m) is located in the immed ate vicinity of the cleft.

Fig. 11 Electron micrograph of a cross sect oned perfusion f xated *ete* showing a venous intercellular cleft (ic) with desmosome

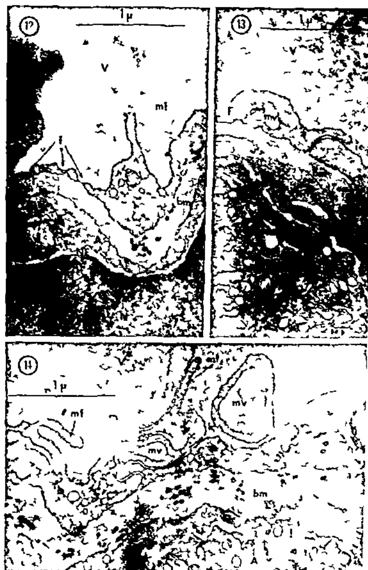


Fig. 1 –14 Electron micrograph of cross-sectioned immersion fixated *etia* showing the possible mechanism behind the formation of vacuoles in the venous endothelial cells A marginal fold (mf) may fuse either with the cell body or with another fold and thus impound droplets of luminal fluid forming a marginal vacuole which then may move towards the central parts of the cell as clear vacuoles. In Fig. 12 and 13 the endothelium is provided with fenestrae (f) The intercellular clefts (ic) in Fig. 13 and 14 appear to have patent gaps The structure of the basement membrane (bm) is very homogenous in all these figures.

The sections shown in Fig. 1– 14 represent a *et* which had been perfused with plasma containing 100 mg % of horse-radish peroxidase (Type VI) The numerous black spots in the cytoplasm and especially in the basement membrane most probably represent focal concentrations of the peroxidase molecules.

actually consisted of many layers of basement membranes containing no or very little of interstitial tissue but this could not be ascertained in the present study At the edge of a pseudopodium of a pericyte the basement membrane was seen to split into two leaflets



Fig 15 Electron micrograph of a cross-sectioned perfusion fixated *rete* showing some structural elements of an angular interstice (AI) Numerous collagenous and elastic fibrils a bundle of non myelinated nerve f bres (nf) and perikarya of venous (vp) and arterial (ap) pericytes The arterial pericyte (and its nucleus) has a crescent shape similar to that of the endothelial cells

which then surrounded the cell Between the endothelium and the pseudopodium of a pericyte the basement membrane was rather thin and often discontinuous thus allowing an intimate contact between the endothelial cells and the pericytes

The adventitial tissue was predominantly confined to the angular interstices In these regions numerous collagenous and elastic fibres and some non myelinated *nerve* fibres were observed (Fig 15) The perikarya of the pericytes were also located in the angular interst

(Fig 4 and 5) These perikarya were seen to make an elaborate system of processes or pseudopodia disposed perpendicular to the long axis of the vessel The arterial and venous pericytes had large dark nuclei The cytoplasm contained numerous dense particles (glycogen?) some pinocytotic vesicles and also large vacuoles

The arterial and venous pericytes could easily be identified (Fig 4) The arterial pericytes were seen to be in intimate contact with the capillary wall being surrounded by the arterial basement membrane along its entire circumference The shape of these cells (and also of their nuclei) was therefore similar to that of the endothelial cells The perikarya of the venous pericytes were seen to have much more irregular outlines and more rounded nuclei Only their pseudopodia were seen to be surrounded by the (venous) basement membranes the perikarya being located freely in the angular interstices

Measurements of the concentrations of ions in retial tissue The concentrations of K^+ and Na^+ were measured in 24 *retia* of different sizes in 2 *retia* measurements of the concentrations of Ca^{++} and Mg^{++} were also included Similar analyzes of these four ions were performed on two different samples of cell sediments from eel blood It should be emphasized however that with our methods the total concentrations of these substances (ionized and non ionized) were measured This would be of no significance to the values obtained for K^+ and Na In the case of the bivalent ions we would have to consider that a significant but small fraction would exist as non ionized molecules within the cells The results obtained are presented in Table II in which the corresponding concentrations in human erythrocytes are quoted

Discussion

The present structural studies demonstrate how the *rete mirabile* is optimally constructed for effective capillary exchange The 2 types of capillaries are arranged so that a maximal surface area is available for the exchange between the arterial and venous system The total capillary surface area in each capillary system is practically identical as was also reported by Krogh (1929) A *rete* of average size having a cross sectional area of 5 mm^2 and a volume of 21 mm has some 34 000 arterial and 22 000 venous capillaries with a total surface area in each capillary system of about 30 cm^2 (Table I)

The capillaries have an almost uniform length of 4 mm The linear relationship between the cross-sectional areas of the *retia* and the corresponding calculated capillary surface areas (Fig. 3) indicated that the capillaries have constant calibres From the values quoted in Table I the mean (inner) diameter of the vessels would be in the range of 9–10 μm the venous capillaries being wider than the arterial ones by a factor of 1 35–1 6 In this respect they are similar to other capillaries investigated in lower vertebrates but wider than mammalian capillaries

The *rete* contains very little of interstitial tissue As has been presented in Eq 1 and as might be accepted from inspection of the micrographs of cross sectioned *retia* the capillary lumina represent about 2/3 and the tissue elements (capillary walls + interstitial tissue) about 1/3 of the cross-sectional area The correspondence between the radii calculated (according to Eq 1) and those measured (Table I) supports this presumption The capillary

walls constitute the major fraction between 1/2–1/3 of the tissue In the *rete* therefore the interstitial tissue would only represent between 1/9 and 1/6 of the cross-sectional area or of the volume

The luminal surface of the endothelial cells especially in the venous capillaries showed microvillous processes similar to those present at the surface of mesothelial cells Microvilli have been noted in the vessels of the pecten of the bird s eye and in the choroid *rete* of fish (Fawcett 1963) but also in mammalian vasculature such as in the rat Gasserian ganglion and testis (Gabbiani and Majno 1969) and in the dog pulmonary artery (Smith *et al* 1971) The presence of microvilli in the capillaries of the *rete* may have an effect on the capillary exchange by making an increase in the capillary surface area and by producing an eddying flow (of cell free plasma?) along the endothelial surface

The pinocytotic or plasmalemmal vesicles appeared to be similar to those of mammalian capillaries as to their size and concentration in the cells (Palade and Bruns 1968 Casley Smith and Chin 1971) In addition to this microvesicular system the endothelial cells con tained a separate system of vacuoles of considerably greater sizes as was also found by Dorn (1961) and Fawcett (1963) Such giant vacuoles have been described in mammalian endothelium by several investigators and are usually interpreted as representing a reaction to cell injury such as mechanical trauma hypoxia freezing, exposure to hyperosmotic solutions *etc* (Luse and Harris 1960 Hoff and Gottlob 1967 Ts ao and Spaet 1967) Different theories have been proposed for the genesis of these vacuoles Mitochondrial swelling autolysis of cytoplasmic content or dilatation of endoplasmatic reticulum However it cannot be excluded that giant vacuoles may originate in a more normal way from plasmalemmal invaginations or more probably from a fuse of cell processes or mar ginal folds impounding droplets of luminal fluid which then move towards the cell center as clear vacuoles The pictures shown in Fig 12–14 are suggestive of such processes resulting in the formation of these vacuoles If therefore this vacuolization of the endothelial cells actually reflects an *in vivo* phenomenon it might represent a special transport activity which could play a significant role in the transcapillary exchange

The width of the intercellular clefts were in the range of 100–300 Å It was an almost constant finding that the clefts showed narrow regions These narrow regions most probably representing the junctions could extend for 100–400 Å in depth In the vicinity of these regions special junctional structures could be observed such as desmosomes with bundles of cytoplasmic fibrils converging on it—similar to those described by Farquhar and Palade (1963) and by Kelly (1966) in epithelia. One could also observe dense plaques which were located at one or both poles of the desmosomes most probably representing attachment devices In the structural studies of the retial vessels earlier performed (Dorn 1961 Fawcett 1963 not quoted by Bendayan *et al* 1974) the junctional lumina were always seen to be obliterated by such plaques This finding would indicate these structures to represent con tinuous attachment bands running along the junction in a direction parallel to the free surface of the endothelium (*zonulae occludentes*) In the present study the major fraction of the junctions in the perfusion fixated *retia* as well as in the immersion fixated *retia* had patent channels with an average diameter of 110–120 Å We would therefore suggest the existence of *maculae* rather than *onulae occludentes* in the retial endothelial cell

tions Attention should also be called to the detection of fine granulated less dense material in many junctions If transcapillary transport actually takes place through the intercellular clefts we would have to consider that the diffusional resistance of this matrix consisting mainly of hyaluronic acid (Laurent 1970) may be different from that of water

The basement membranes contained numerous microfibrils and often appeared as a layered structure In general the basement membranes of capillaries have been described as having an homogenous structure However the presence of distinct zones of different translucency has been described in intestinal capillaries of rats (Clementi and Palade 1969) and in lung capillaries of rabbits (Hovig *et al* 1971) after a treatment with EDTA sufficient to give formation of edema In our studies no signs of edema could be detected not even in those preparations which had been perfused with Ringer solutions prior to the fixation

The structure of the endothelial cells of the arterial capillaries were found to differ essentially from that of the venous endothelial cells In the arterial vessels the endothelial cells were thick marginal folds were lacking and they had a continuous plasmalemma The venous endothelium was more attenuated the number of pinocytotic vesicles was considerably smaller numerous marginal (cytoplasmic) folds could be detected and the plasmalemma was provided with clustered fenestrae Thus the arterial capillaries seemed to be very similar to those of mammalian skeletal muscles whereas the venous vessels resembled some types of visceral capillaries in mammals as for instance those of the intestinal mucosa (Bennett *et al* 1959)

This dimorphism between the two sets of capillaries in the *rete* indicate the permeability properties of their walls to be significantly different Intestinal capillaries have been shown to be more permeable than those of skeletal muscles the former allowing molecules of larger sizes to penetrate (Vogel and Strocker 1967) and having higher filtration coefficients (Folkow *et al* 1963) It appears reasonable to presume therefore that the arterial capillary walls will represent the greatest resistance to transcapillary diffusion in the *rete*

According to the theory generally accepted the diffusion of water soluble molecules across the capillary walls takes place through the intercellular clefts (Landis and Pappenheimer 1963) Whether the junctions of skeletal muscle capillaries actually have patent gaps or not has been discussed (Karnovsky 1968 Bruns and Palade 1968 a) According to Karnovsky most of the junctions do represent open channels with a minimal width of 40 Å

The fact that visceral capillaries are more permeable than capillaries of the continuous type both types having junctions with nearly the same structure and dimensions may indicate that the fenestrae represent an additional pathway to transcapillary transport In fact Bruns and Palade (1968 b) and Karnovsky (1968) have shown that a considerable fraction of peroxidase or ferritin molecules appears to pass through the fenestrated regions of the endothelium However the number of fenestrae in the venous capillaries of the *rete* was found to be significantly smaller than what has been observed in mammalian visceral capillaries In for instance the renal peritubular capillaries the fenestrae may take up as much as one fifth of the total area (Friederici 1968) Accordingly then the venous capillaries in the *rete* could perhaps be expected to be less permeable than mammalian intestinal capillaries but more permeable than mammalian skeletal muscle capillaries

These considerations about the permeability properties of the observed capillaries are

of course highly speculative The structure of the capillary walls as it appears in the sections may thus not reflect the situation *in vivo* Essential changes in structure qualitative as well as quantitative may take place during the fixation.

The present studies indicate the capillary endothelial cells to be highly differentiated ones The endothelial cells of the *rete* were seen to contain a system of vacuoles as well as one of pinocytotic vesicles These findings could indicate the participation of the endothelial cells in transcapillary transport While transcellular transport by vacuoles may be a speculative hypothesis the existence of transport through pinocytotic vesicles seems to be well established Macromolecules such as ferritin (r = 55 Å) have been shown to be transported in such a way indicating that the micro-vesicular system of endothelial cells may represent the large pore system of non fenestrated capillaries and lacteals (Bruns and Palade 1968 b Dobbins and Rollins 1970 Casley Smith and Chin 1971) It has also been established that the major transcapillary transport of water in the *rete* takes place through the endothelial cells (Stray Pedersen and Steen 1975)

Bendayan *et al* (1974) have made very interesting suggestions about the morphological and functional significance of the pinocytotic vesicles observed in capillary endothelial cells These investigators used fixatives containing dimethyl sulphoxide and acrolein substances which are known to contribute to good preservation of membrane structure (Sandborn 1966 and 1970) They assumed the vesicles (and vacuoles?) which appear as isolated structures on micrographs to represent sectioned parts of tortuous channels or tubules which are continuous throughout the cell According to these investigators alternate widening and narrowing of such tubules may indicate that they exhibit some sort of peristaltic activity It should be emphasized that a transcapillary transport through such a system of contractile tubules may be considerably more rapid and require less metabolic energy than transport via pinocytotic vesicles It is also noteworthy that the diameter of the dilated parts of the tubules found by these investigators could be as great as 1300 Å a value which conforms precisely to the pore dimensions calculated on the basis of permeability data of the retial capillaries (Stray Pedersen and Steen 1975)

As seen from Table I the tissue content of K^+ is significantly smaller and the content of Na significantly higher than that of homologous blood cells and human erythrocytes The ratio between the concentrations of K^+ and Na^+ was found to be close to unity As for Ca^{++} and Mg^{++} the content of these substances is very similar to that of homologous cells but considerably higher than that of human red cells which are extremely poor in Ca^+ These values for the ionic content of retial tissue however should be considered as rather rough estimations since no attempts were performed in order to estimate the fraction of intra vascular fluid which might not be removed by squeezing and/or air inflation of the organ The significance of the presented ionic concentration of retial tissue is not clear If the endothelial cells are actually so poor in K^+ one would expect the electrochemical potential of their membranes to be smaller than and the permeability properties perhaps essentially different from that of other cell membranes

Studies on the permeability properties of the retial capillaries have been based on measure ments of the exchange between the arterial and venous system (Stray Pedersen and Steen 1975 Stray Pedersen 1975) The actual barrier would therefore comprise two structurally

different capillary walls as well as the interposed tissue elements As seen from Fig. 4 this barrier would vary considerably in thickness as well as in composition along the circumference of neighbouring vessels At the levels of the angular interstices the arteriovenous barrier may have a thickness of several μ consisting mainly of layers of interstitial tissue and cells A transcapillary transport of any significance through these regions would therefore seem very unlikely The other extreme is represented by those regions offering *minimum resistance to transcapillary diffusion in which the barrier consists of following* layers only Arterial endothelium arterial and venous basement membranes (partially fused) and venous endothelium Such regions however are very unfrequently encountered and represent maximally 5% of the total exchange surface In most places a pseudopodium of a pericyte and smaller amounts of interstitial tissue and fluid are seen to be imposed between the basement membranes It seems reasonable to suggest that the major part of the transcapillary exchange takes place across this part of the barrier which comprises about 50% of the circumference of the capillaries In conclusion then the functional barrier" in the *rete mirabile* would consist of two outer layers consisting of arterial or venous endothelium with their basement membranes and a middle adventitial layer of a thickness of about 1 μm The average thickness of this functional barrier was found to be 1 6 μm the area constituting about 50% of the total capillary surface area

Both of us have been Research Fellows of the Norwegian Research Council for Science and the Humanities Financial support from this Council and from the Norwegian Council on Cardiovascular Diseases is gratefully acknowledged We also wish to thank Prof J B Steen for all help and advice during the performance of this work and Prof B A Waaler and Prof L Jorgensen for their valuable comments on the manuscript Thanks are also due to Miss N Brewer and Mrs S Schram for skilful technical assistance

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Acta physiol scand 1975 94 358–367
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Integrated Somatomotor, Cardiovascular and Gastrointestinal Adjustments Induced from the Cerebellar Fastigial Nucleus

By

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Received 10 January 1975

Abstract

Lisander B and J Martner *Integrated somatomotor cardiovascular and gastrointestinal adjustments induced from the cerebellar fastigial nucleus* Acta physiol scand 1975 94 358–367

Behavioural cardiovascular and gastric responses induced by fastigial stimulation were observed in conscious cats with gastric fistulas indwelling fastigial electrodes and arterial catheters Fastigial stimulation elicited oral behaviours e g grooming and chewing together with tachycardia and pressor responses while gastric motility was unaffected in most cases as was gastric hydrochloric secretion In subsequent experiments on the anesthetized animals it was found that the same fastigial area could suppress the intestino-gastric inhibitory reflex Fastigial influences on small intestinal motility were investigated in anesthetized cats, well recovered from surgical isolation of intestinal loops whose motility could therefore be recorded without Fastigial stimulation either depressed or did not influence ileal motility before laparotomy but after this procedure excitatory responses were uniformly recorded This reversal is explained by a fastigial suppression of inhibitory intestinal reflexes elicited by the laparotomy

Stimulation of the rostral fastigial pole elicits a powerful pressor response (Achari and Downman 1969 Miura and Reis 1969) and in unanesthetized cats or rats complex behavioural responses including grooming biting and eating (Reis Doba and Nathan 1973 Berntson Potolicchio and Miller 1973 Ball Micco and Berntson 1974) This fastigial pressor area may also modify several autonomic mechanisms controlling gastrointestinal motility in anesthetized cats (Lisander and Martner 1974 1975) The question arises which gastrointestinal influences that are elicited in unanesthetized cats since cerebellar autonomic responses can differ markedly in conscious and anesthetized animals (Rasheed Manchanda and Anand 1970) Second are the behavioural responses associated with specific patterns of gastric function in terms of motility and secretion? Are the changes in behaviour secondary to the visceral adjustments? To elucidate such problems experiments were performed in conscious cats where chronically implanted fastigial electrodes could be stimulated and the effects on gastric volume or secretion could be followed

Concerning intestinal motility the fastigial influence is mainly excitatory in anesthetized cats It has been proposed that this reflects a suppression of the sympathetic intestino- intestinal

inhibitory reflex (Lisander and Martner 1974 Martner 1975) a term here also used for inhibitions elicited by the inevitable trauma from opening the abdomen If so then quite different fastigial effects on intestinal motility might be revealed if instead a less traumatic abdominal preparation is used Therefore the second part of this study was devoted to atraumatic intestinal motility recording on isolated intestinal loops with the ends emerging through the abdominal wall in animals previously operated and completely recovered

Methods

The experiments were carried out on 17 animals of either sex All operations were performed under Nembutal® (Abbott) anesthesia (30-40 mg/kg b w i p) with endotracheal intubation and aseptic precautions Before the animals ate postoperatively saline was given subcutaneously A mixture of streptomycin and penicill n (Streptocillin® Novo 0 5 g+ 00 000 IU) was injected daily for 5-14 days After the various operations the animals recovered rapidly and appeared undisturbed within one week All cats except two exceeded their preoperative weight at the end of the experimental period

Gastric fistula op ation

The abdomen was opened in the midline An incision was made in the stomach close to the major curvature A plexiglass cannula (inner diameter 14 mm) was fitted into the opening and the end of the cannula was brought out through a stab wound on the left s de of the abdomen The midline opening was closed in layers The an mals were allowed to eat on the second or third day after the operation These an mals were three weeks to two months later implanted with fastig al electrodes and an arterial catheter

I ol t on of an intestinal segment (6 cats)

The abdomen was opened in the midl ne A segment of jejunum or ileum 10-15 cm long was isolated keeping the mesenteric nervous and vascular supply intact The rest of the intestine was anastomosed end to end The ends of the isolated segment were brought out through two stab wounds on the right side of the abdomen The serosa was sutured to the skin and the midl ne open ng was closed in layers The animals were allowed to eat from the day after the operation

Implantation of a terial cathete and ce ebella el ctrodes (8 cats)

The neck was incised and the right carotid artery cannulated by a silicon rubber catheter (Silastic Dow Corning inner diameter 0 76 mm) with the tip in the aort c arch The other end of the catheter was connected to a P 3 AC transducer writ ng on a Grass polygraph

Then the head of the an mal was placed in a Horsley-Clarke apparatus The skin was incised and trephination was made in the skull over the cerebellum Electrodes made from teflon coated platinum-iridium wire (Medwire Corp wire diameter 0 3 mm) were inserted bilaterally in the fast gial pressor areas at an angle 15 posterior to the ertical plane The optimal location of the electrodes was judged by the blood pressure response to test stimulat ons They were then fixed in position by dental cement and connected to a micro plug provided with a stainless steel hook The plug was hooked to the bone through a burr hole nd f xed w th cement The hook was later used as the posit ve electrode The free end of the blood pressu e catheter was drawn under the skin to the opening in the scalp The catheter was connected to a small valve (see below) screwed to the bone after wh ch the skin wounds were closed The valve and the m croplug projected through the skin The arterial catheter was kept open by daily flushings with heparin sol tion — In three additional an mals only arter al catheters were implanted

Obs rvations in the anesthet ed a m ls

The an mals beha ioural responses to fastigial stimulation were observed in a box w th a one way m rror at least 5 days after the electrode implantation A polyethylene tube with a connector was screwed to the valve on the head The al e was opened by th s procedure The arterial pressu e was reco ded by a Grass P23 AC transducer

Gastric juice was collected by rinsing the stomach with 0 ml of sal ne ia a short tube through th gastrostomy Titration was done with 0 1 M NaOH with phenolphthaline as the indicator To increase gastri

secretion, histamine chloride (Vitrum) was injected subcutaneously. Gastric volume was recorded by the balloon method and a Grass force transducer with the animal kept in a small cage. The intraluminal pressure was kept at 5–10 cm H_2O (Lisander 1975, Lisander and Martner 1975).

Acute experiments on anesthetized animals

All cats were chloralosed, 50–60 mg/kg b.w. i.v. To eliminate any artifacts due to somatomotor changes diallyl-bis-nor-toxoferine-dichloride (Alloferin® Roche) 0.1 mg/kg i.v. was given. Artificial respiration was then maintained. In the group with chronic implanted fastigial electrodes stimulation was performed via these electrodes while electrodes were acutely inserted in the other cats. Square wave pulses at intensities from 0.02 to 0.2 mA were used (in one cat 0.6 mA). Pulse duration was usually set at 1 ms and the impulse frequency at 50 Hz (Lisander and Martner 1975).

Gastric motility was recorded as described above, though the intragastric balloon was inserted via the esophagus in the cats without a gastric fistula. *Intestinal motility* was recorded as pressure changes in a balloon inserted into the isolated intestinal loop. Distending pressures (100–200 cm H_2O) could also be applied to this loop in order to elicit an intestino-gastric inhibitory reflex.

Histological procedures

After the acute experiments the heads of the animals were perfused by saline followed by a 10 % solution of formaldehyde. The cerebellum and adjacent brainstem structures were then paraffin embedded, sectioned in slices of 15 μm and stained by the Nissl technique. In some experiments the cerebellum was frozen sectioned.

Results

1. *Conscious cats with fastigial electrodes*

Cats with a gastric fistula were later implanted with chronic fastigial electrodes, thus allowing for simultaneous observation of behaviour and gastric motility or acid production. The results from these animals are summarized in Table I.

Behaviour. Although all electrodes gave marked pressor responses, some differences in electrode site could not be avoided. However, the similarities between behaviour patterns in different cats were remarkable, although the stimulation thresholds could vary considerably between the cats. If the electrode tip was placed in the rostral ventromedial fastigial pole 0.02 mA was sufficient to elicit responses, while in one cat, where the electrode tip was placed 0.5 mm caudally to this area, 0.5–0.6 mA was necessary to elicit the same response. The character of the responses was determined partly by stimulation intensity. Thus, at

TABLE I. Observations from 7 cats during fastigial stimulation. Abbreviations and signs: E, Eating. G, Grooming. 0, No influence. –, Inhibition. Gastric motility refers to the situation in the unanesthetized animal and the influence on the IGIR (intestino-gastric inhibitory reflex) was tested in the terminal experiment.

Cat no	Behaviour	Gastric motility	Influence on IGIR
1	E G	0	–
2	G	0	–
3	G		
5	G	–	–
6	G	0–	0
7	G	0	–
9	G	0	

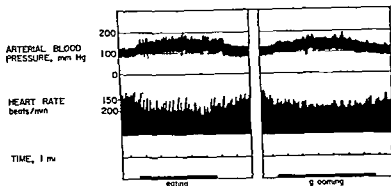


Fig. 1 Conscious cat 3 0 kg with an indwelling arterial catheter Note that spontaneous eating and grooming are associated with increased blood pressure and heart rate

threshold intensity sporadic licking of the mouth appeared If the stimulation was prolonged the cat began licking its fur At slightly increased intensity the cat usually pulled at the fur with its front teeth The grooming in most cases started with either front paw and then progressed to other regions if stimulation was prolonged With increasing intensity the grooming became more and more vigorous The responses generally started immediately after the onset of stimulation and ceased within five seconds after the stimulus In the animals reported here they were entirely reproducible throughout the experiment and could be repeated in a later test

The animals had free access to water milk and minced fish together with a few large rubber corks Only in one cat stimulation caused eating together with grooming Very often especially at higher intensities the animals interrupted their grooming and started biting at the cardboard covering the bottom of the box Pieces were torn away but not ingested If instead a rubber cork was located very near the animal could take a few bites on it

No clear signs of aggression were noted and the animals could be handled even during intense stimulation without danger They only interrupted their stereotype behaviour for a few seconds if the box was opened and they were touched during stimulation If another cat was put into the cage the stimulated animal behaved as before during stimulation even if it displayed hostility towards the intruder between stimulations

The somatomotor response pattern was thus stereotyped, but performed in a well co-ordinated fashion with no ataxia or other motor disturbances No signs that the stimulations might be irritating or painful such as growling, hissing or attempts to escape were noted

The thresholds for the blood pressure increase and for the above described behaviour were identical whether current or impulse frequency was varied Spontaneous grooming was paralleled by marked increases in blood pressure and heart rate (Fig. 1) The same observation was repeatedly made in three other cats implanted with arterial catheters only

Gastric motility While stimulation in conscious cats caused pronounced effects on blood pressure and behaviour changes in gastric motility were seldom demonstrated In only 2 cats prompt inhibitions of gastric tone were elicited resembling adrenergically

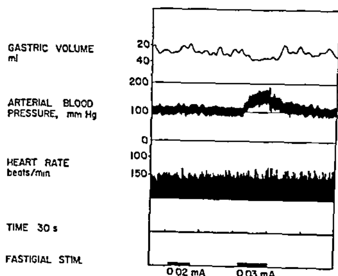


Fig. 2. Conscious cat 2.7 kg. Fastigial stimulation (50 Hz 1 ms) at 0.02 mA produces no response while at 0.03 mA blood pressure rise and inhibition of gastric motility are elicited together with grooming and gnawing on card board.

relaxation in acute experiments on anesthetized cats (Lisander and Martner 1975). This response had the same threshold as the blood pressure response (Fig. 2).

Gastric HCl secretion. The results from cats tested for fastigial influence on gastric HCl production are shown in Fig. 3. The mean prestimulatory values are somewhat higher than basal HCl secretion in cat 0.01–0.05 mEq per hour (Emås 1960). Fastigial stimulation did not change HCl secretion while subsequent submaximal histamine administration increased acid output.

2. *Anesthetized animals*

a) *Gastric motility.* In subsequent experiments performed on the anesthetized animals fastigial stimulation produced either slight inhibitory (4 cats) or biphasic gastric responses (1 cat). However following laparotomy the only response pattern obtained was that of

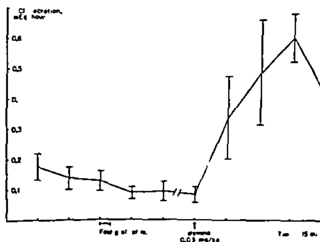


Fig. 3. Compiled results from recordings of HCl secretion in 5 cats taken at 15 min interval. Bars indicate S.E. After the intersection n=4 as one cat was omitted from the histamine test. Note that fastigial stimulation (30 s each minute for 5 min) does not produce any significant change in HCl secretion.

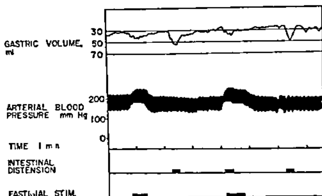


Fig. 4 Cat 3 1 kg with isolated ileal segment Chloralose anesthesia, Alloferin® 0 1 mg/kg b w i v artificial respiration The vagal nerves cut and the distal ends stimulated (6 Hz, 1 ms 8 V) Ileal distension produces gastric relaxation. Note that a concomitant fastigial stimulation (50 Hz, 1 ms 0 05 mA) which in itself produces relaxation, totally abolishes the gastric response to intestinal distension.

excitation The latter kind of response was still present after vagotomy provided that the distal ends of the cut vagal nerve were stimulated but disappeared following guanethidine administration This denotes fastigial inhibition of the intestino-gastric inhibitory reflex (Lisander and Martner 1975)

b) *Gastric motility responses to intestinal distension* If a distending pressure was applied to the isolated intestinal loop an intestino-gastric inhibitory reflex could be elicited provided a vagal tonic activity was present Concomitant fastigial stimulation abolished or markedly reduced this sympathetically mediated reflex (Fig 4) although fastigial stimulation alone had

Fig 5 Cat 2 5 kg, isolated ileal segment. Chloralose anesthesia A Fastigial stimulation (50 Hz, 1 ms, 0 1 mA) produces inhibition of ileal and gastric motility B After bilateral vagotomy the ileal inhibition persists while the gastric response is abolished C Following laparotomy and efferent vagal stimulation (5 Hz 1 ms 5 V) both the stomach and the ileum display excitatory responses

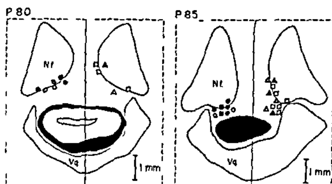


Fig 6 Cerebellar frontal sections 8 and 8 5 mm posterior to the interaural line Results from conscious cats are shown to the left and responses from anesthetized cats to the right Electrode sites in one cat at P 7 5 and in another at P 9 0 are projected to P 8 0 and 8 5 respectively Filled circles Oral behaviour Open circles No influence on gastric motility in spite of oral behaviour Filled squares Inhibition of gastric motility Open squares Inhibition of the intestino gastric inhibitory reflex Open triangles Inhibitory intestinal responses before laparotomy Filled triangles Excitatory intestinal response after laparotomy Nf Nucleus fastigius Vq Ventriculus quartus

a slight relaxatory influence on the stomach Even pronounced fastigially induced gastric relaxations could be changed into excitations following laparotomy as illustrated in Fig 5

c) *Ileal motility* In intact cats fastigial stimulation did either not influence ileal motility or as depicted in Fig 5 panel A produced an inhibition of spontaneous ileal contractions still present after vagotomy (panel B) However after laparotomy dep essing ileal motility fastigial stimulation enhanced the motility in all 4 cats during the entire stimulation period (panel C) The latter fastigial response did not require intact vagal nerves but could be locked by guanethidine

d) *Jejunal motility* Two cats had isolated jejunal segments As in the other cats jejunal distension caused gastric inhibition which was suppressed by concomitant fastigial stimulation Motility in the jejunal segments was either unaffected or slightly inhibited by fastigial stimulation Laparotomy depressed jejunal peristalsis but only transiently and no clear excitatory responses to fastigial stimulation were present after this procedure

Electrode positions

Histological examination confirmed that the effects were elicited from the fastigial pressor area *i e* the ventromedial rostral fastigial pole In Fig 6 the different kinds of responses from the conscious cats are shown on the left Electrode sites and the character of the responses from the experiments on the anesthetized cats are depicted to the right

Discussion

Stimulation of the anterior pole of the fastigial nucleus elicited in the conscious cats grooming biting chewing and occasionally eating confirming findings by Reis *et al* (1973) and Berntson *et al* (1973) Similar oral responses have also been observed in rats (Ball *et al* 1974) Zanchetti and Zoccolini (1954) reported that fastigial stimulation could

trigger sham rage in decorticated cats Reis *et al* (1973) also reported aggressive [illegible] as a result of fastigial stimulation which however was not observed in the present [illegible] When they were exposed to fastigial stimulation they showed little interest in [illegible] environment and started instead gnawing and tearing at the nearest suitable [illegible] *per se* well coordinated manner but did not as is typical for hypothalamically [illegible] responses explore the surroundings in search of appropriate goal objects [illegible] present cerebellar responses took place only during stimulation whereas [illegible] feeding responses are less stimulation bound (*cf* Ball *et al* 1974) Actual [illegible] during fastigial stimulation was only seen in one of the animals

Even though stimulation thresholds and electrode positions differed [illegible] the cats the similarities in the animals concerning their fastigially induced [illegible] striking Thresholds for the autonomic and behavioural responses were [illegible] suggesting that the stimulated fastigial pressor area might induce an [illegible] autonomic and somatomotor adjustments This possibility is also [illegible] that spontaneously elicited grooming behaviour was paralleled by [illegible] increases in blood pressure and heart rate thus resembling the responses [illegible] stimulation A conspicuous finding in the unanesthetized animals was [illegible] of specific gastric motility changes associated with the oral behavioural [illegible] stimulation somewhat surprising since experiments on anesthetized [illegible] strated that the fastigial nucleus is quite potent in modifying gastric [illegible] Martner 1975) Further none of five unanesthetized cats tested [illegible] responses and all displaying grooming biting and chewing showed [illegible] HCl secretion in association with these stimulations Had a truly [illegible] been evoked in connection with these fastigial responses one would [illegible] to take place since it is known that anticipation of eating [illegible] hypothalamic stimulation etc can increase gastric secretion (*e g* [illegible] Mason and Nelsen 1969)

This lack of regularly occurring stimulation bound gastric [illegible] animals may suggest that the particular grooming and chewing [illegible] stimulation is not primarily elicited by *e g* afferent impulses from [illegible] involved in feeding behaviour On the other hand the mentioned [illegible] various well integrated behavioural responses to stimulation of [illegible] also induce complex cardiovascular and gastrointestinal ad[illegible] these phylogenetically old cerebellar parts control not only [illegible] also complex behavioural patterns normally initiated from higher [illegible] further studies are needed before such a hypothesis can be [illegible]

When the same animals were used for acute experiments [illegible] stimulation caused the same gastric motility responses as [illegible] Martner 1975) as long as the animals were not exposed to [illegible] inhibitory or occasionally biphasic responses The former [illegible] inhibition of motility and the latter by a combination of [illegible] and an augmented vagal cholinergic activity (Lisander [illegible] soon as the animals were exposed to laparotomy [illegible]

sympathetic intestino-intestinal (intestino-gastric) inhibitory reflexes fastigial stimulation elicited only excitatory gastric responses These were due to a fastigial suppression of the mentioned spinal inhibitory reflexes induced by laparotomy (*cf* Lisander and Martner 1975)

In the animals with chronically isolated intestinal loops pronounced adrenergically mediated, intestino-gastric inhibitory reflexes could be induced by distension of these loops (*cf* Piercy and van Liere 1926 Youmans 1968) Fastigial stimulation substantially reduced these reflex inhibitions of gastric motility As long as the abdominal cavity was left intact fastigial stimulations alone produced as mentioned gastric inhibitory or biphasic responses and, in the chronically isolated intestinal loops either no appreciable response or motility inhibition. After laparotomy only excitatory responses were encountered in the ileal loops while the jejunal loops (two cats) showed no or very weak excitatory responses This is in agreement with the effects of fastigial stimulation in acute experiments on laparotomized cats, where gastric and ileal responses are uniformly excitatory while the jejunum displays a mixed response pattern (Lisander and Martner 1975 Martner 1975) The present findings thus support the view that the fastigial nucleus and hence the cerebellum, exerts control of gastrointestinal motility predominantly though not only by suppression of the sympathetically mediated intestino-gastric inhibitory reflexes whenever such spinal reflex arcs are activated (Lisander and Martner 1975 Martner 1975) The weaker effects on the jejunal loops might simply be due to a less intense adrenergic impact on this part of the gut (Kewenter 1965)

The present results concerning neurogenic gastrointestinal control also demonstrate the importance of the experimental situation for the outcome of the effects In studies of [illegible] motility in general abdominal surgery is often inevitable thus eliciting the [illegible] spinal inhibitory reflexes. These can, in turn, be suppressed by structures [illegible] the medullary "depressor area" (Johansson, Jonsson and Ljung 1965) and from the cerebellum according to the present results and probably from other parts of the central nervous system as well Evidently it is important for the correct interpretation of results concerning nervous control of gastrointestinal function first to define clearly the experimental circumstances involved. For example depending upon whether or not the preparation employed has led to an activation of the spinal sympathetic inhibitory reflexes results of different experimental studies may be strikingly different and apparently contradictory

Thus, the present study has shown that restricted parts of the cerebellar fastigial nucleus can induce well-integrated behavioural patterns associated with complex adjustments of the cardiovascular and gastrointestinal systems. Evidence is accumulating to indicate that the cerebellum, besides its wellknown coordination of simple somatomotor movements controls also visceral and emotional functions as well as certain complex behavioural patterns (*e g* Zanchetti and Zoccolini 1954 Berntson *et al* 1973 Reis *et al* 1973 Dow 1974 Ball *et al* 1974) The results of the present study are in agreement with such a concept.

This study has been sponsored by grants from the Swedish Medical Research Council (No B 4-14X 16-10C) from Svenska Sällskapet för Medicinsk Forskning and from the Faculty of Medicine University of Göteborg.

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Acta physiol scand 1975 94 368-377

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Effects on Gastric Motility from the Cerebellar Fastigial Nucleus

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Received 10 January 1975

Abstract

LISANDER B and J MARTNER *Effects on gastric motility from the cerebellar fastigial nucleus* Acta physiol scand 1975 94 368-377

In acute experiments on chloralosed cats gastric motility blood pressure and heart rate were investigated for influences exerted by the fastigial nucleus Besides pressor responses fastigial stimulation could produce either gastric excitation or relaxation and the background of these responses was analysed by selective nerve sectioning and administration of suitable autonomic blocking agents Suppression of prevailing gastric motility was found to be mediated mainly by increased discharge in adrenergic nerve fibres but also by adrenal catecholamine release — Gastric excitation could be induced in three different ways first by increased activity in vagal cholinergic fibres second by fastigial suppression of the vago vagal non adrenergic relaxatory reflex In addition when laparotomy or other noxious abdominal stimuli had induced inhibitory gastric reflexes the consequent sympathetic discharge could be suppressed by fastigial stimulation resulting in enhanced gastric motility — The importance of background activity in the various nervous pathways for the fastigially induced gastric responses is discussed

Central nervous control of gastric function has been extensively investigated and is well documented Gastric motility may be influenced from various parts of the central nervous system such as certain cortical structures hypothalamus and brain stem (for details see Thomas and Baldwin 1968) Also the cerebellum seems to be involved in the control of gastric function although only few studies have been carried out to elucidate this matter Bard *et al* (1947) reported that motion sickness in dogs is abolished by extirpation of the uvula and nodulus Wolfe (1969) showed that gastric ulceration sometimes follows restricted cerebellar lesions in cats Gastric motility can be influenced from the anterior lobe in rabbits (Ban *et al* 1956) and from the vermis and the fastigial nucleus in cats (Manchanda Tandon and Aneja 1972)

In general most visceral functions can be modified from the cerebellum and the fastigial nucleus seems to be especially potent in giving autonomic responses Thus powerful pressor responses can be elicited by electrical stimulation of the ventromedial part of the rostral fastigial pole (Achari and Downman 1969 Miura and Reis 1969) However this area can influence also other autonomically innervated organs *e g* the intestinal tract (Lisander and Martner 1974 Martner 1975 a) and the urinary bladder (Martner 1975 b) The findings

presented in these studies indicated that an augmentation of intestinal motility could be induced by a fastigial suppression of the adrenergically mediated intestino-intestinal inhibitory reflex. This reflex inhibits motility throughout the gastrointestinal tract following distension of any part of the intestine (cf Pearcy and Liere 1926 Youmans 1968) Besides intestinal distension any noxious stimulus to the abdomen including laparotomy depresses gastrointestinal motility and this is here included in the term intestino-intestinal inhibitory reflexes

Studies on the stomach offer certain advantages in the further investigation of cerebellar influences on this type of reflex which below will be referred to as the intestino-gastric inhibitory reflex as far as the stomach is concerned In the first place it is possible to record gastric motility without laparotomy a procedure which inevitably elicits the intestino-gastric inhibitory reflex Second background cholinergic tone which is necessary for demonstrating this reflex (Jansson and Martinson 1966 Jansson and Lisander 1969) is much easier to control in the stomach *e g* by graded efferent vagal stimulation than in the intestines which usually display more irregular intrinsic activity The present study was undertaken to investigate the fastigial influence on gastric motility and if such an influence could be demonstrated to analyse the nervous mechanisms involved

Methods

43 cats of either sex deprived of food for 24 h were used for the experiments After induction with ether anesthesia was maintained by i v administration of chloralose 50–60 mg/kg b w For stereotaxic stimulation the head of the animal was fixed in a Horsley Clarke apparatus Following trephination and partial removal of the tentorium, sharp monopolar stainless steel electrodes were inserted perpendicularly to the stereotaxic horisontal plane Square wave pulses were delivered by a constant current stimulator at intensities varying from 0 05 to 0 3 mA corresponding to a voltage range of 1–4 V Pulse duration was set at 1 ms and the impulse frequency usually at 50 Hz after recordings of the frequency–response relationship at 10 to 60 Hz. Decerebration was performed at the intercollicular level either with a blunt spatula or by using high frequency electric coagulation by a stereotaxically inserted needle electrode

After each experiment a small lesion was made around the cerebellar electrode tip by anodal direct current of 1 mA for 10 s The head of the animal was then perfused with saline followed by a 10 solution of formaldehyde In 1/3 of the cats the cerebellum and adjacent brain stem structures were paraffin embedded sectioned in slices of 15 μm and stained by the Nissl technique In the rest of the material the cerebellum was frozen sectioned and mounted without staining In the latter way the precise location of the intercollicular lesion was determined as well

Intestino-gastric inhibitory reflexes were elicited either by laparotomy or by repeated injections of 0 1–1 ml of acid (0 1 M HCl) or alkali (0 1 M NaOH) through the abdominal wall. The vagal nerves were dissected free in the neck and often cut in the course of the experiment. The distal ends were then placed on bipolar ring electrodes for graded efferent stimulation. When instead the vago-vagal gastric relaxatory reflex was studied one vagal nerve was left intact but the other one cut with the central end mounted on a bipolar electrode (for details see Jansson 1969 a) for afferent stimulation (10– 0 Hz, 1 2 ms and 6–15 V) In other cats arrangements were made for reversible blockade of vagal transmission by temporary nerve cooling at the neck level Spinal cord transection was performed between C_6–C_7 or between C_7–Th_1

In cats subject to laparotomy adrenal secretion was eliminated by encircling ligatures around both glands Adrenocortical substitution was then given by i v injection of hydrocortisone (Solu-Gluc® Erco) 10 mg/kg. To allow for graded baroreceptor stimulation in some experiments one carotid sinus region was partly isolated and via a polyethylene tubing connected to one of the femoral arteries A sigma motor perfusion pump could change the pressure in the sinus within wide limits pressure being measured from a side branch close to the sinus by means of a P23 AC transducer In these experiments the contralateral sinus nerve and also the vagal nerves were sectioned thus eliminating other baroreceptor stations.

Arterial pressure was measured through a femoral catheter connected to a Statham P23 AC transducer writing on a Grass polygraph and heart rate was recorded by a Grass Tachograph unit Gastric motility was measured as volume changes in a large intragastric waterfilled balloon inserted into the stomach via the esophagus Via a large caliber tube the balloon was connected to a volume reservoir whose weight was continuously recorded by a Grass force displacement transducer FTO3 The water level was adjusted to obtain a constant intragastric pressure of 5–10 cm H_2O (for details see Jansson 1969 a)

Gallamine iodide (Flaxedil®) 4 mg/kg b w i v or diallyl bis-nor toxoferine-dichloride (Alloferin® Roche) 0 1 mg/kg b w i v was often used to eliminate any artifacts due to somatomotor changes Artificial respiration was then maintained by a respiratory pump Pharmacological adrenergic blockade was induced by guanethidine (Ismelin® CIBA) 4–5 mg/kg b w i v in some cats in combination with phentolamine (Regitin® CIBA) 3 mg/kg b w i v and propranolol (Inderal® ICI) 0 5–1 mg/kg b w i v For cholinergic blockade atropine (atropine sulphate) 0 5–1 mg/kg b w i v or methylscopolamine (Skopyl® Pharmacia) 0 015–0 03 mg/kg b w i v were used

Results

Fastigial stimulation was found to influence gastric motility in various ways Background gastric tone which to a great extent depends on the preparation procedures was an important factor in determining the response pattern Therefore the experiments were subdivided into different groups depending on the initial operation and subsequent experimental arrangements

1 *Fastigial influence on gastric motility in intact cats*

Out of a total number of 23 cats in this group 11 displayed either increased or decreased gastric motility while the remainder showed a biphasic response pattern In the latter group the sequence of the responses was always the same *i e* an initial suppression followed by an increased gastric tone even if the net response in some cats was dominated by inhibition and in others by excitation If the latter group was added to those cats exhibiting uniform citation and the former group included in the total number of cats displaying inhibition ach group contained about the same number of cats

Gastric relaxations induced by fastigial stimulation were either abolished or substantially reduced by parasympathetic blockade Thus atropine (1 cat) or vagotomy (4 cats) eliminated the response while the latter procedure in 3 other cats substantially reduced suppression of gastric motility If the distal ends of the cut vagal nerves were stimulated gastric vagal tone could be reestablished artificially and it was then again possible to demonstrate fastigially induced gastric relaxation After administration of the adrenergic blocking agent guanethidine (7 cats) the gastric relaxations were abolished in 3 cats but persisted in 4 cats though reduced and delayed in onset suggesting a hormonal mechanism The disappearance of gastric relaxation after adrenergic blockade is illustrated in Fig 1 Spinal cord transection in 2 cats displaying a biphasic gastric response abolished the inhibitory phase while the excitation persisted Surprisingly in one other cat spinal cord transection failed to abolish gastric relaxation which was instead blocked by atropine

Excitatory gastric responses were completely abolished by atropine (2 cats) or by vagotomy (4 cats) while spinal cord transection failed to do so (4 cats) In the spinal cats however the gastric contractions were abolished by administration of atropine or methylscopolamine as is illustrated in Fig 2 Also evident from this figure is the abolition of fastigially induced tachycardia

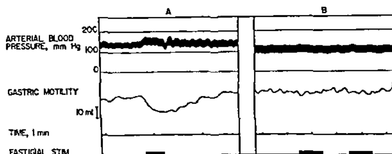


Fig 1 Cat 2 4 kg right vagal nerve cut. Fastigial stimulation (50 Hz, 1 ms 0 3 mA) induces a prompt gastric relaxation together with a blood pressure rise (panel A) After administration of guanethidine 4 mg/kg b w i v., both the gastric and the blood pressure responses are abolished (panel B)

2 *Fastigial influence on gastric motility in cats subject to laparotomy*

Laparotomy invariably induced a profound adrenergically induced depression of gastric tone by the intestino-gastric inhibitory reflex Independent of the type of fastigial response obtained before laparotomy the response pattern following this procedure was always that of excitation The total number of cats in this group amounted to 14 if 5 cats receiving intra abdominal injections of acid or alkali (see later) were included Bilateral vagotomy eliminated or reduced the excitatory responses to fastigial stimulation However the responses could always be restored or even potentiated if the distal ends of the cut vagal nerves and the fastigial nucleus were concomitantly stimulated Fig 3 clearly illustrates the importance of background vagal cholinergic activity Fastigial stimulation in the absence of vagally induced gastric tone evokes no response while there is a substantial increase in gastric tone when fastigial stimulation is performed during concomitant vagal stimulation usually the more so the higher the frequency of vagal stimulation up to about 6 Hz. — If time was allowed to elapse after vagotomy stomach motility often returned without vagal stimulation

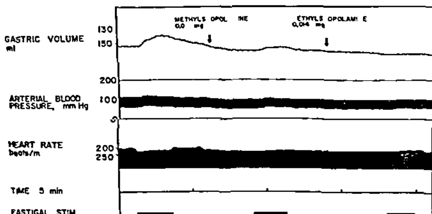


Fig 2. Cat 3 6 kg the spinal cord transected between C_7 and Th Fastigial stimulation (50 Hz, 1 m 0 1 mA) produces an e citatory gastric response which is eliminated by subsequent adm nistration c methylscopolamine

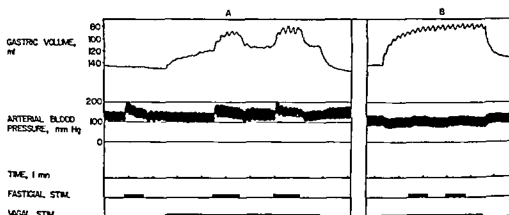


Fig 3 Cat 3 1 kg vagotomized and laparotomized with adrenals ligated A Fastigial stimulation (50 Hz, 1 ms 0 2 mA) with and without efferent vagal nerve stimulation (6 Hz 2 ms 8 V) Note that gastric responses are only elicited against a background of vagal activity B When adrenergic inhibitory discharge is blocked by guanethidine 4 mg/kg i v even low frequency vagal stimulation (4 Hz) induces the same level of gastric excitation that required a combined vagal and fastigial stimulation in A Moreover no fastigial effects can now be demonstrated

and it was then again possible to enhance this motility by fastigial stimulation Administration of acid (HCl) or alkali (NaOH) also induced a transient intestino gastric inhibitory reflex which could be abolished by guanethidine When gastric tone was reflexly inhibited in this way fastigial stimulation again induced excitatory gastric responses evidently mediated by adrenergic mechanisms since they were eliminated by guanethidine (4 laparotomized cats and one injected with acid)

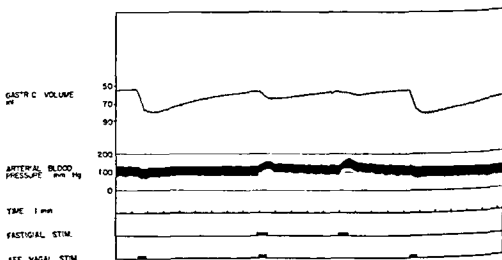


Fig. 4 Cat 3.2 kg adrenergic blockade (not complete which is evident from the remaining blood pressure rise) with guanethidine (5 mg/kg) phentolamine (3 mg/kg) and propranolol (1 mg/kg) Cholinergic blockade with atropine, 1 mg/kg. The vago-vagal relaxatory reflex is elicited by afferent vagal stimulation (14 Hz, 2 ms, 8 V) and produces a profound depression of gastric tone Note that concomitant fastigial stimulation (50 Hz, 1 ms, 0 1 mA) inhibits this reflex

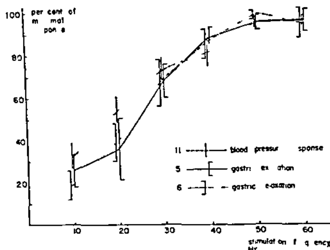


Fig 5 Frequency–response curves for fastigial stimulation compiled from 11 cats comparing blood pressure rise excitatory and inhibitory gastric responses. The excitations are in these cats due to suppression of the intestino-gastric inhibitory reflex while gastric inhibitions are produced by increased adrenergic discharge Note that all 3 curves follow a similar course Bars indicate S E.

The excitatory gastric responses in laparotomized cats remained after decerebration (3 expts)

The fastigially induced gastric effects were not secondary to baroreceptor reflexes since the motility responses were not influenced by artificially varied carotid sinus pressure in three animals

3 *Fastigial influence on the vago-vagal nonadrenergic gastric relaxatory reflex*

Electrical stimulation of the central end of one cut vagal nerve with the other left intact regularly induced profound and longlasting gastric relaxations that persisted after both adrenergic and cholinergic blockade (Jansson 1969 a) In 7 out of 9 cats treated with atropine and adrenergic blockade (6 cats treated by guanethidine and 3 by spinal cord section) it was possible to inhibit this vago-vagal gastric relaxation by fastigial stimulation The range of fastigial inhibition varied from merely changing the speed of the gastric relaxation to almost complete blocking of the reflex (Fig 4) In this experiment fastigial stimulation *per se* induced a modest but prolonged gastric relaxation probably due to adrenal catecholamine release and if this relaxation is taken into account (third signal in Fig 4) the fastigial suppression of the vago-vagal reflex seems to be nearly complete

4 *Stimulus–response relationships*

The fastigial pressor response displays a gradually rising frequency–response curve from 10 Hz up to 50 Hz which in most experiments was the optimal frequency level When compared with the frequency response curves for the fastigially induced gastric inhibitions and excitations respectively they show a conspicuous congruence in virtually all respects (Fig 5) No difference in threshold could be observed for these three types of responses if instead current was varied

5 *Responsive cerebellar structures*

Almost all gastric responses were elicited from the fastigial pressor area. As seen from Fig. 6 showing three frontal sections 8 8 5 and 9 mm posterior to the interaural line the signs

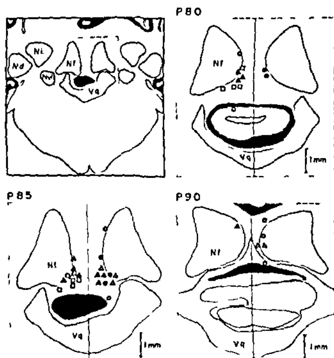


Fig 6 Frontal sections 8 8.5 and 9 mm posterior to the interaural line For the sake of clarity the same types of gastric responses are always gathered on the same side of the drawings Open circles No response Open triangles Gastric relaxations Open squares Suppression of intestino-gastric inhibitory reflex Filled triangles Gastric excitatory responses Filled dots Suppression of the vago vagal inhibitory reflex Nf nucleus fastigius N I Nucleus interpositus N d Nucleus dentatus V q Ventriculus quartus N v l Nucleus vestibularis lateralis

representing effective stimulation points are localized in the rostral ventromedial part of the fastigial nucleus and adjacent cerebellar white matter anatomically corresponding to the fastigial pressor area In only 3 cats clearcut excitatory gastric responses were obtained in absence of a pressor response all points localized dorsally to the pressor area It was not possible demonstrate any anatomic separation between points giving different types of gastric responses they were all intermingled and moreover from the same electrode position both excitatory and inhibitory gastric responses could be elicited in different phases of the experiment depending on *e g* operative procedures

Discussion

Gastric motility was found to be influenced by the fastigial pressor area in a most complex way Thus in the anesthetized but otherwise intact animals either increases or decreases in gastric tone could be elicited and biphasic responses were often seen with an initial suppression followed by excitation Concerning the excitatory gastric responses to fastigial stimulation seen in these intact animals they were at least in part mediated via the vagi and of cholinergic nature since they were eliminated or greatly reduced by vagotomy or anticholinergic agents but not by spinal cord section

The neurogenic sympathetic inhibitory influence on gastric motility is predominantly exerted by means of inhibitory connections with the intramural cholinergic excitatory neurons (*e g* Jansson and Martinson 1966) This was taken into account when the inhibitory gastric responses to fastigial stimulation were analysed They were abolished by cholinergic

blockade or by vagotomy but reappeared if the peripheral ends of the cut vagi were stimulated simultaneously with the fastigial pressor area

After guanethidine the inhibitory responses were either abolished or reduced In the latter case they appeared with a delay of about 20 s indicating a hormonal mechanism in all likelihood adrenal catecholamine release It is known that guanethidine does not prevent adrenal catecholamine release whose peripheral effects are instead potentiated (Abercrombie and Davies 1963) These findings clearly indicate that the relaxations from fastigial pressor area stimulation in *intact* animals were adrenergically mediated

Laparotomy profoundly changed the gastric responses to fastigial pressor area stimulation Noxious stimuli to the abdominal cavity including laparotomy intestinal distension *etc* induce a marked adrenergically mediated depression of gastric motility *via* intestino-gastric inhibitory reflexes Independent of the direction of fastigially induced gastric responses seen before laparotomy the animal always displayed excitatory gastric responses after this procedure

Such excitatory gastric responses might be explained either by increased activity in vagal cholinergic fibres by an inhibition of adrenergic discharge or by a suppression of prevailing activity in the vagal relaxatory fibres (Martinson 1965) Again the gastric excitatory responses were abolished by vagotomy or in some cases reduced In the laparotomized cats it was possible to increase gastric tone by fastigial stimulation even after bilateral vagotomy provided that the distal ends of the vagal nerves were simultaneously stimulated but these gastric excitatory responses were abolished by guanethidine This indicates that the excitatory responses seen in the laparotomized cats were due to fastigial suppression of a prevailing sympathetic influence on the stomach This situation should be compared to that before laparotomy where fastigial stimulation led to an increased sympathetic activity to the stomach in about half the animals Evidently laparotomy caused an activation of the intestino-gastric inhibitory reflex and this reflex is inhibited from the fastigial pressor area Further when transient intestino-gastric inhibitory reflexes were induced by intraabdominal injections of irritant solutions fastigial stimulation led to a gastric motility increase by suppressing this transient reflex while the same fastigial stimulation produced motility inhibition as long as basal gastric motility was present It is known that the intestino-intestinal inhibitory reflex can be suppressed from supraspinal levels *e g* from the medullary depressor area (Johansson Jonsson and Ljung 1965 1968) The arterial baroreceptors were on the other hand of no importance for the excitatory gastric responses induced by fastigial stimulation in laparotomized animals even though they are activated along with the fastigial pressor response

The cerebellum has been found to influence also hypothalamically induced autonomic responses (Lisander and Martner 1971 1973) and diencephalic lesions (Sawyer Hilliard and Ban 1961) or precollicular decerebration (Ban *et al* 1956) abolish some autonomic responses elicited from the cerebellum On the other hand the fastigial pressor response persists after midbrain transection (Miura and Reis 1969 Achari and Downman 1970) while fastigially induced effects on intravesical pressure were changed (Manchanda and Bhattarai 1972) In the present study the fastigial inhibition of the intestino-gastric inhibitory reflex persisted after intercollicular decerebration indicating that the effects w

mediated via structures cranial to the midbrain It is possible that the fastigial inhibition is mediated via the medullary depressor area which as mentioned can suppress the spinally conveyed intestino intestinal (gastric) reflexes

The possibility that the fastigial nucleus might increase gastric tone also by suppressing the discharge in the vagal relaxatory fibres was investigated in connection with their activation in vago-vagal reflexes It was clearly demonstrated that this reflex could be inhibited by fastigial stimulation This suppression was not exerted at the effector level by *e g* adrenergic or cholinergic mechanisms since the inhibition was still present after combined cholinergic (atropine) and adrenergic blockade (guanethidine or spinal cord transection) The vago-vagal relaxatory reflex is relayed in the lower brainstem since it is unaffected both by intercollicular decerebration and spinal cord transection at C_1-C_2 (Jansson 1969 b) Evidence for a relay center for this reflex close to the obex has been presented by Nakazato and Ohga (1971) and the fastigial influence is likely to be exerted at this bulbar level

Histological examination revealed that the cerebellar area from which gastric responses could be elicited was quite restricted (Fig 6) Although the intention was primarily to investigate the fastigial pressor area considerable parts of the fastigial nucleus and adjacent cerebellar areas were stimulated before the pressor area was identified However it was a regular finding that the gastric effects were almost exclusively obtained from the fastigial pressor area There were no signs of any gross anatomical differentiation between the points causing different types of gastric responses The technique utilized however does not allow for a more detailed analysis of the neuron pools involved due to current spread *etc* The frequency-response curves (Fig 5) representing the fastigially induced blood pressure rise the gastric relaxation in intact animals and the suppression of the intestino gastric inhibitory reflex all follow a similar course suggesting that the neuron pools responsible for these ects operate through functionally similar mechanisms

The results indicate that the fastigial pressor area can induce gastric relaxation by increased adrenergic sympathetic discharge and also by adrenal catecholamine release Increased gastric tone can on the other hand be elicited *via* inhibition of the sympathetic intestino-gastric inhibitory reflex by increasing vagal excitatory cholinergic discharge and also by inhibition of reflexly induced activity in the vagal relaxatory fibres Neurogenic control of the stomach is most complex and exerted at several levels Thus intramural neurons give rise to a basic activity which is modified by intramural ganglionic by spinal sympathetic and by bulbar vagal reflexes Several parts of the central nervous system not the least hypothalamic and cortical sections are able to influence gastric motor activity The question arises whether these higher autonomic centres exert their effects mainly or only by modifying the activity in reflexes affecting stomach motility In the present experiments evidence was obtained indicating that the cerebellar fastigial nucleus is able to suppress two autonomic reflexes inhibiting the stomach Further if gastric motor activity was high as in intact animals the fastigial nucleus often lowered this basal (cholinergic) activity by inducing an increased sympathetic activity perhaps by modulation of spinal sympathetic reflex arcs In any case the present study reveals that the cerebellum tends to increase gastric tone when it is low and decrease it when it is high The results thus also stress the importance of the prevailing experimental conditions during explorations of central nervous control of autonomic mechanisms

This study has been sponsored by grants from the Swedish Medical Research Council (No B74-14X 16-10C) from Svenska Sällskapet för Medicinsk Forskning and from the Faculty of Medicine University of Göteborg.

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1971 Inuchijima 1973), it is often assumed that the increased resistance is maintained almost solely by neurogenic mechanisms also in the "established" phase of SHR hypertension

Since however MAP is a product of cardiac output (CO) and total peripheral resistance (TPR) the true significance of the pressure fall subsequent to complete cardiovascular denervation can be evaluated only if both the CO and TPR components are known. In fact, perfusion experiments reveal that resistance is higher in established SHR hypertension than in NCR even during maximal vasodilatation (Folkow *et al* 1970), reflecting the presence of hemodynamically important structural changes of the SHR resistance vessels (*cf* Folkow *et al* 1973 1974) Furthermore studies on isolated perfused SHR hearts show that they display a smaller stroke volume than NCR hearts at a given diastolic filling pressure mainly due to the left ventricular hypertrophy in SHR and the consequent decrease in average myocardial diastolic stretch (Hallback, Isaksson and Noresson 1975).

Against such a background there are good reasons to believe that TPR may be elevated but CO decreased after denervation in SHR inspite of a "normalized" blood pressure compared with equally treated NCR. It was therefore decided to study more precisely the relationship between MAP CO and TPR after complete cardiovascular denervation by means of pithing in SHR and NCR.

Methods

30 6–7 months old SHR body weight ranging between 300 to 370 g, and 30 age- sex and weight-matched NCR were used After anesthesia with Inactin® (5-aethyl-5 (1-metyl-propyl)-2 thiobarbituric acid Promonte), 100 mg/kg b wt., a cannula was introduced into the aortic arch via the carotid artery The cannula was connected to a pressure transducer and a Grass polygraph for recordings of blood pressure and heart rate and subsequently used for withdrawal of arterial samples. One jugular vein was cannulated by a PE 10 catheter the tip of which was placed close to the heart. The trachea was cannulated and the animals were artificially ventilated at a rate which barely suppressed spontaneous respiration. A rythmic inflation was used with a peak pressure of 9–10 cm H_2O which was equally maintained in all animals before and after pithing. It should here be noted that the positive pressure breathing tends to reduce CO by impediment of venous return, particularly after cardiovascular denervation. Flaxedil, 1 mg 100 g was given i.p to suppress spontaneous motility and seizures in combination with the pithing procedure Pithing was performed by introducing a [illegible] mm steel rod from the atlanto-occipital joint down the entire spinal canal to destroy the spinal medulla, thereby eliminating all sympatho-adrenal activity

CO was measured either by 1) the radioactive rubidium (^{86}Rb) injection technique, whereby also an approximate estimation of CO distribution may be obtained (Sapirstein 1958), or by 2) the cardiogreen dye dilution technique as modified for rats.

1) *CO measurement with the ^{86}Rb technique* was performed on 20 SHR and 20 NCR. 10 pairs were used before and 10 pairs after the standardized pithing procedure. When pressure and heart rate were stabilized, the arterial cannula was connected to an oil-filled, constant speed withdrawal system. Exactly 20 μCi of ^{86}Rb was quickly injected by a step dispenser syringe (0.1 ml) into the jugular catheter After the ^{86}Rb injection, carotid arterial blood was withdrawn at a constant speed of 0.67 ml/min over exactly 10 s. 50 s later the animal was suddenly killed by an i.v injection of 0.4 ml concentrated KCl solution

For CO determination the radioactivity in the 10 s arterial sample was counted by means of a γ-counter Number of counts in weighed samples of kidneys, heart, skeletal muscle skin and liver were used to approximate blood flow through these test organs. Even though the total ^{86}Rb amount does not enter the intracellular "sink" of the respective tissues during the first passage, there is no reason to assume that the amount of trapping in different animals groups would be different. Thus, this technique allows a fair estimation also of CO distribution in experimental animals. The ^{86}Rb method has, however the obvious disadvantage of allowing only one CO measurement per animal. Consequently measurements were first performed on one pair of SHR and NCR with intact sympathetic control, followed by another pair in which the measurements were performed during steady state condition after pithing.

2) *CO measurements with the dye dilution technique* allowed multiple determinations in each animal and therefore only 10 SHR and 10 NCR were used At least two measurements were performed both before and after pithing Subsequent to the two measurements after pithing most of the rats received papaverine 9 5 mg/kg in order to ensure maximal vasodilatation prior to a final CO measurement

By means of a step dispenser syringe 0 04 ml of cardiogreen solution was injected into the jugular vein Arterial blood (maximally 0 35 ml *per* recording) was withdrawn at a constant speed of 0 67 ml/min passing through a specially designed low volume (0 05 ml) densitometer cuvette After the determination the blood was reinfused The densitometer cuvette was connected to a Servogor recorder for recording of the dye dilution curve

Calibration of the densitometer was performed with known concentrations of dye diluted in human plasma and the system was linear over the range of the recording scale The accuracy of the dye dilution determinations was checked against actual plasma flows in a tube system with a mixing chamber and a pump delivering constant flows from 10 up to 90 ml/min This check revealed a linearly increasing over estimation of the flow with the dye dilution technique as compared to the actual flow in the system, being maximally 9 per cent at flow rates of 90 ml/min For calculating CO from the dye dilution recordings, a *digitizer unit connected to a Hewlett Packard calculator was used*

Results

Mean arterial pressure (MAP) in SHR 198±4 mm Hg was significantly higher than in NCR 132±4 mm Hg ($p<0\,001$) these pressure levels being somewhat above the usual since the carotid artery cannulation implies a partial baroreceptor unloading Table I presents mean values of cardiac output (CO) total peripheral resistance (TPR) and heart rate (HR) before and after pithing the former two parameters being expressed *per* 100 g of b wt There was no appreciable difference in CO values obtained by the two methods and the results are therefore pooled in the Table During the prevailing experimental conditions involving artificial positive pressure ventilation and unilateral carotid occlusion by

TABLE I Mean arterial pressure (MAP) cardiac output (CO) total peripheral resistance (TPR) and heart rate (HR) before and after pithing in SHR and NCR Values presented are expressed as means ±S E. Levels of significance and per cent difference between SHR and NCR are given below * indicates significant difference ($p<0\,001$) from control situation

	Control conditions				After pithing			
	MAP mm Hg	CO ml min 100 g	TPR PRU/ 100 g	HR beats/ min	MAP mm Hg	CO ml/min 100 g	TPR PRU/ 100 g	HR beats/ min
SHR n= 0	198±4	18 9+1 0	11 0+0 6	348±7	40±[illegible]	9 ?±0 9	4 9±0 4	*269±9
NCR n=.0	13 ±4	4 0 . 0 9	5 6±0 2	358±9	44±2	14 1±0 9	3 6±0 3*	63±6
Level of sign difference SHR-NCR	p<0 001	p<0 001	p<0 001	n s	n.s	p<0 001	p<0 01	n s
Per cent difference SHR-NCR	+51	-21	87	—	—	-35*	+36	—

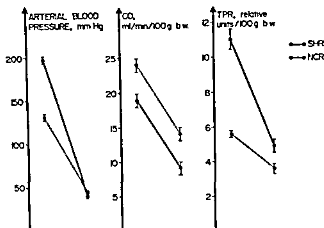


Fig. 1 Illustrates the fall in arterial pressure (left part) cardiac output (CO) (middle part) and total peripheral resistance (TPR) (right part) before and after pithing in SHR and NCR Vertical bars indicate S E

the arterial cannulation CO was about 20 lower in SHR than in NCR in the control resting state ($p < 0.001$) while TPR was nearly twice as high in SHR as in NCR ($p < 0.001$)

After pithing the arterial pressures fell to about equally low levels in the NCR and SHR groups The CO difference was even more pronounced after pithing CO being some 35 per cent lower in SHR than in NCR ($p < 0.001$) the same being true for stroke volume since HR were about the same In return TPR was now about 35 per cent higher in SHR compared with NCR ($p < 0.01$) It should be noted that after pithing, eliminating neurogenic cardiovascular control and drastically lowering pressure vascular smooth muscle tone was virtually eliminated as checked by injection of papaverine which did not appreciably further reduce TPR from the postpithing values Therefore the systemic vascular beds of NCR and SHR could be considered maximally dilated in the prevailing situation and TPR is then only dependent on the structural design of the resistance vessels and on the transmural pressures which were largely equal in NCR and SHR —The results are also presented in a diagrammatic form in Fig 1

In the group of SHR and NCR studied with the cardiogreen technique the percentual fall in CO as a result of pithing could be determined in each animal Compared with NCR a *modestly though significantly greater CO reduction was observed in the SHR group* ($p < 0.05$) where CO was reduced by 63 ± 5 per cent upon pithing while the CO reduction in NCR was 54 ± 2 per cent

Measurements of blood flow distribution in SHR and NCR by the ^{86}Rb technique revealed that before pithing the cutaneous and renal blood flows were somewhat lower in SHR, while in skeletal muscle myocardium and liver the flow was about equal in SHR and NCR This general pattern also prevailed after pithing even though the muscle blood supply appeared to increase modestly in both groups

In summary even after pithing and papaverine administration TPR was some 35 per cent higher in SHR than in NCR while CO and stroke volume was about 35 per cent lower in SHR Consequently MAP reached about the same low levels in SHR and NCR when all nervous influences on the cardiovascular system were abolished

Discussion

The importance of the cardiovascular neurohormonal control system for the initiation and maintenance of high blood pressure in genetically hypertensive rats of the Okamoto strain (SHR) and also of the Smirk strain (GHR) has been repeatedly emphasized (*e g* Okamoto 1969 Smirk 1970 Nosaka *et al* 1972) and many studies provide evidence of an increased neurohormonal discharge in SHR (*cf* Okamoto 1969) Further also young prehypertensive SHR display an inherent evidently genetically linked hyperreactivity of central autonomic structures resulting in exaggerated increases of heart rate and arterial pressure compared with NCR whenever enhanced alertness is displayed or when the rats are exposed to "mental stress in their daily life (Hallback and Folkow 1974) Moreover the effects of immunosympathectomy in newborn GHR and SHR (Clark 1971 Folkow *et al* 1972) the beneficial effects of early treatment with sympathicolytic drugs (Weiss 1974 Weiss Lundgren and Folkow 1974) and of longterm reduction of normal environmental stimuli (Hallback 1975) strongly suggest that central neurohormonal mechanisms are of crucial importance for the very initiation of hypertension in these animal models of human essential hypertension (*cf* Folkow 1975)

Against such a background the finding that almost equally low arterial pressure levels are reached in SHR and NCR after complete cardiovascular denervation has often led to the apparently logical suggestion that the increased pressure in SHR must be essentially the result of an increased sympathetic activity also in the established phase of hypertension (Okamoto 1969 Shibayama Mirogami and Sokabe 1971) However arterial pressure depends not only on total peripheral resistance (TPR) but also on cardiac output (CO) Consequently a recording only of the product of these two parameters *i e* of mean arterial pressure (MAP) does not allow any conclusions concerning the mechanisms behind the pressure fall after autonomic blockade Iriuchijima (1973) recently measured both MAP and CO before and after ganglionic blockade in 9 pairs of SHR and NCR which were younger (as an average 17 weeks old) than the present ones As in the present study arterial pressures reached about equally low levels after denervation TPR appeared to be higher in SHR than in NCR, although no statistical significance was obtained possibly because of the fairly small material

The present results were obtained on a much larger experimental material and two different techniques for CO measurements were used, which did not mutually differ to any significant extent After pithing, CO was found to be about 35 per cent lower in SHR than in NCR (p 0 001) and the same was true for stroke volume since heart rates were largely the same In return TPR was largely 35 per cent higher in SHR ($p < 0\ 01$) so that MAP was about equal in NCR and SHR. Before these results are further considered the methods used for CO measurement will be briefly evaluated The ^{86}Rb technique may be slightly influenced by the modest recirculation that occurs even though this tracer is to a marked degree "trapped" in the tissues during its first circulation This technique will also tend to overestimate very low flow values since the standardized sampling period was relatively short (10 s) and it will further not allow for extrapolations as will the cardiogreen technique Therefore the CO values after pithing may if anything, be slightly overestimated.

and the TPR ones correspondingly underestimated especially in SHR where CO was then particularly low

A possible disadvantage of the cardiogreen method would be the unavoidable and slightly larger blood loss during each arterial sampling (0 35 ml at most) compared with the ^{86}Rb technique However this blood loss corresponds to only some 1 5 per cent of the total blood volume in rats of this size Neither heart rate nor pressure revealed any significant cardiovascular influence of this small blood withdrawal which was moreover quickly re infused to the animal Therefore the CO methods used seem to be satisfactory for the present purpose

A standardized positive pressure ventilation was as mentioned maintained under iden tical circumstances in SHR and NCR throughout the experiments Such artificial respira tion is likely to induce cardiovascular reflexes by the impediment with venous return which tends to reduce enddiastolic filling pressure Such interferences not present during normal conditions might to a different extent influence CO in NCR and SHR Thus con trol CO was about 20 per cent lower in SHR than in NCR in the prevailing situation while no such difference is found when anesthetized SHR and NCR are compared without arti ficial respiration (own preliminary results)

After spinal destruction eliminating all cardiovascular reflex adjustments the systemic vessels became virtually maximally dilated as checked by papaverine TPR is in this situa tion essentially determined by the structural design of the resistance vessels and by their prevailing transmural pressure which was largely equal in NCR and SHR Therefore the 35 per cent higher TPR after pithing compared with NCR ($p < 0\ 01$) indicates a structural narrowing of the SHR resistance vessels in agreement with previous findings in artificially perfused maximally dilated systemic vascular bed in SHR and NCR (Folkow *et al* 1970) Further hemodynamic analyses indicate an increase in wall/lumen ratio confined to the *pre*capillary resistance vessels and mainly due to a rapidly established media thickening (Folkow *et al* 1973 1974) Due to this precapillary structural autoregulation in SHR both excitatory and inhibitory influences result in exaggerated precapillary luminal changes for given changes in smooth muscle activity An important positive feedback interaction is thus created between functional excitatory and structural influences so that a higher resistance may be maintained in SHR by the same neurogenic activity as in NCR (*cf* Fol kow 1975) However it also implies exaggerated resistance and pressure reductions in SHR compared with NCR when as in this particular case all neurohormonal excitatory influences are suddenly eliminated (Fig 1)

Further the present results show a relatively more pronounced CO reduction in SHR than in NCR upon complete denervation In this situation cardiac performance is mainly determined by the myocardial heterometric and homeometric autoregulation (*cf* Sarnoff and Mitchell 1962) Since MAP was largely equal in NCR and SHR after pithing the dia stolic filling pressure will primarely determine CO since inherent heart rates proved to be largely equal in NCR and SHR in this case Thus stroke volume was about 35 per cent lower in SHR than in NCR after pithing which may in part be explained by a relatively more pronounced interference with the SHR myocardial blood supply as a result of the mar fall in pressure head More important is probably the fact that SHR left ventricles

a lower stroke volume for a given enddiastolic pressure compared with NCR The reason is that the average myocardial stretch becomes reduced largely in proportion to the left ventricular hypertrophy shifting the Frank Starling curve for SHR to the right in the physiological range of filling pressures (Hallback Isaksson and Noresson 1975) Consequently the sympathetic control of the low pressure capacitance compartments might become increasingly important along with the development of SHR hypertension being here needed to ensure a proper filling of the gradually less compliant left ventricle

In summary the hemodynamic pattern in SHR and NCR after complete cardiovascular denervation in the prevailing situation associated with maximal systemic vasodilatation is in SHR characterized by a 35 per cent higher TPR and a proportionally lower CO and stroke volume compared with equally treated NCR These opposite changes in TPR and CO after denervation lead to about equal MAP levels in SHR and NCR Both these changes are consequences mainly of the rapidly established structural adaptation of the cardiovascular high pressure compartments occurring in hypertension and involving not only the left ventricle and systemic arteries but also the precapillary resistance vessels

This study was partly supported by grants from the Swedish Medical Research Council (No B74-14X 16-10C) from the Medical Faculty University of Göteborg and from Svenska Nationalföreningen mot Hjärt och Lungsjukdomar AB Hässle generously covered part of the expenses for a technician

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Acta physiol scand 1975 94 386–392
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Rapid Transport of Noradrenaline in Adrenergic Axons of Rat Sciatic Nerve Distal to a Crush[1]

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Received 15 January 1975

Abstract

HAGGENDAL, J A DAHLSTRÖM and P A LARSSON *Rapid transport of noradrenaline in adrenergic axons of rat sciatic nerve distal to a crush* Acta physiol scand 1975 94 386–392

The transport of NA in the rat sciatic nerve distal to a crush was studied in 5 mm segments The disappearance of a NA fraction in consecutive segments with time after crushing (0–12 h) was interpreted to indicate a proximo-distal migration of a NA fraction into further distal parts of the nerve This transportable NA fraction was found to be about 45 of the NA in normal sciatic nerves and migrated at a rate of approximately 8 mm/h The apparently non mobile fraction (55) was probably mainly located in vaso-constrictor nerve terminals of the blood vessels supplying the nerve This non mobile NA fraction does not contribute to the NA accumulations proximal to a crush Thus when calculating the rate of transport of any substance from accumulation experiments corrections for a non mobile fraction must always be considered

In adrenergic neurons noradrenaline (NA) storage granules are transported intra axonally towards the nerve terminals (*cf* Dahlstrom and Häggendal 1966 Banks Mangnall and Mayor 1969) Following a nerve crush NA rapidly accumulates in the adrenergic axons on the proximal side of the crush Most of the accumulated NA is probably stored in amine storage particles as shown by the reserpine-depletability (Dahlström 1965 1967 a) Furthermore the synthesis and break-down of NA in the nerve segment just above the crush are balanced (McLean and Keen 1972) Therefore the time-course curve for NA accumulation has been used to calculate the rate of transport of amine storage granules Thus the NA has been used as a marker for the storage particles Using this procedure the rate of transport in rat sciatic nerve axons was found to be about 5 mm/h (Dahlström and Häggendal 1966) The calculations were however based on the assumptions that a) all the NA in the sciatic nerve was transportable and that b) the amine granules stored a constant amount of NA during their transport and accumulation

[1] Reported in part at the Wenner-Gren Symposium on "Dynamics of Degeneration and Growth in Neurons" Stockholm, May 1973

An alternative method to estimate the rate of transport of NA storage granules would be to follow the clearance of NA from the nerve segments just distal to a crush It has been demonstrated (*e g* Ochs and Ranish 1969) that rapid intra axonal transport of labelled proteins proceeds *at the same rate in axons distal to a crush as in intact axons Since* the amine storage particles are transported with the rapid phase this should hold true also for these organelles This study was undertaken to study the rate of amine granule transport distal to a crush and also to investigate the proportion (fraction) of transportable NA in this system

Material and Methods

Male albino rats *of the Sprague-Dawley strain were used Under ether* anesthesia the sciat c nerves were crushed bilaterally *a m* Lubińska (1959) as described earlier (Dahlström 1965) The animals were killed after 0 1 2 3 6 and 12 h and the sciatic nerves were d ssected out and cut into eight 5 mm segments, relative to the crush 2 segments above the crush and 6 segments below were usually removed except in one series of 3 h crushes where one segment above and seven below were dissected Uncrushed nerves were used as controls

The NA *content in the different sections* was assayed *accord ng to the trihydroxyindole method* as modified by Häggendal (1963) For every assay the corresponding sections were pooled from generally 10 rats (*i e* ?0 nerve parts) In every experimental series 100 ng of NA was added to one sample and the reco ery of NA during the estimation procedure was calculated The recovery was as a mean 83° which was corrected for

In some experiments the NA content of the connective tissue sheath (epi and perineurium) was assayed in normal and 6 h crushed nerves Two cm d stal to the crush (except for the 0 5 cm immediately distal to the crush) was dissected out and placed on a cool glass plate The connective tissue was carefully stripped off the nerves and the sheath and the content (axons) were assayed for NA content separately The same procedure was used for the corresponding segment of normal nerves The sheath and the content were also stud ed by fluorescence histochemistry according to the method of Hillarp and Falck (for ref and description see Corrodi and Jonsson 1967) The tissue was spread on glass plates air-dried, treated with paraformaldehyde and examined as described previously (*e g* Malmfors 1965) Photographs were taken with green sensitive Scopix G film (Gaevert) with exposure times ranging between 60–90 s

Results

The NA content in the respective nerve segments (A–H) did not differ in normal and 0 h crushed nerves (Table I) The NA content in 1–12 h crushed nerves are shown in Fig 1

TABLE I The d stribution of NA in different segments (A–G) of rat sciatic nerve relati e to the site of crushing in normal (uncrushed) and 0 h crushed nerves Mean ± S E. are given in ng/5 mm nerve Numbers in brackets represent number of observations (The normal values represent 100 for the respective segments in Fig 1) S te of crush between A and B The most distal *segment is G For each estimation* ⌞*0 nerves were pooled.*

Segment	A	A	↓	B	C	D	E	F	G
Normal	1 30 ±0 084 (10)	1 07 ±0 043 (10)		1 05 ±0 049 (10)	1 1⌞ ±0 043 (10)	1.29 ±0 047 (10)	1 19 ±0 075 (10)	1.25 ±0 055 (8)	1.54 ±0 103 (7)
0 h	1.35 ±0 003 ( )	1 09 ±0 22 ( )	↑	0 94 ±0 01 (2)	1 07 ±0 06 (?)	1 10 ±0..0 ( )	1.09 ±0 06 (2)	1.29 ±0 05 (2)	1.53 ±0 15 ( )
	← proximal		crush			distal →			

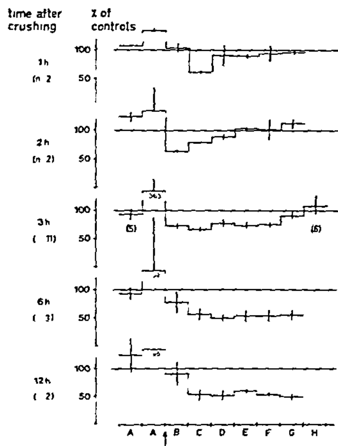


Fig. 1 The NA content in 5 mm segments of the rat sciatic nerve 1–12 h after crushing. Segments A¹ and A proximal to the crush, segments B H distal to the crush (arrow) Means ± S E. are given in of the normal values of the respective segment (see Table I). There is no quantitative evidence for a retrograde transport in segment B 2 h *post* crushing. The gradual rise later could be caused by a small retrograde accumulation but may also be due to contamination from segment A at the dissection.

where the results are expressed in per cent of the values of the corresponding control segment As shown previously the NA content in segment A just above the crush increased rapidly with time (Fig. 1)

In the part distal to the crush (Fig. 1) a clearcut decrease could be seen already after 1 h in segment C After 2 h the decrease involved segments B and C with a tendency to some decrease also in segment D Three h after crushing the NA decrease was seen in all distal segments except the 2 most distal ones (G and H) involving 5 segments distal to the crush In the 6 segment (G) there was in one series of experiments (3 h, I) a clearcut decrease, while in another series (3 h II) no decrease was found All segments showed a decreased NA content 6 and 12 h after crushing, to about 55% of normal (C–G) In segment B the NA content was normal at 1 h low at 2 h and gradually rising between 2 h and 12 h after crushing (Fig. 1)

Since it was of interest to study where the remaining 55 % of NA was located the connective tissue sheath and the nerve fibre core were assayed separately The results are shown in Table II The 2 cm nerve part assayed corresponds to the segments C–F in Fig. 1 Part B was omitted because of the possibility that retrograde accumulations (*cf* Dahlström 1965) could interfere with the results About 22% (0 5 ng) of the NA within a normal 2 cm

TABLE II The NA content (ng per 2 cm of nerve) in the connective tissue sheath and in the core of axon bundles respectively of rat sciatic nerve The dissections were made on an ice-cold glass plate as indicated in the text. Uncrushed nerves (normal) and nerve segments distal to a 6 h crush were used n s indicates not significantly different from normal values

Experiment no	Normal		6 h crush		Decrease in % of normal	
	Sheath	Core	Sheath	Core	Sheath	Core
1	0 48	1 66	0 46	1 14	4	31
2	0 45	1 72	0 33	1 14	27	34
3	0 58	1 88	0 39	1 02	33	46
Mean value	0 50±0 04	1 75±0 07	0 39±0 04 (n s)	1 10±0 04 (p<0 005)	21	37
Total (sheath + core)	2.25 ±0 10		1 49 ±0 06 (p<0 005)		35±5 1	

segment was present in the connective tissue sheath Distal to a 6 h crush about 35% (0 4 ng) of the total NA content (sheath + core) was found in the sheath The content of the core (axons and some connective tissue) was significantly decreased by 37% 6 h after crushing A small decrease was also noticed in the sheath but this decrease was not statistically significant

The histochemical studies revealed the presence of a large number of adrenergic NA containing nerve terminals in blood vessels of the connective tissue sheath (Fig 2) A few *non terminal axons were also observed In the core* a few vessels with adrenergic nerve terminals were observed situated in the thin connective tissue of the endo- and perineurium between the myelinated and non myelinated axons

Since it appeared impossible to totally separate nerve terminals in the connective tissue from axons in the sciatic nerve the quantitative studies in Table II were not extended

Discussion

The results of the present study (Fig 1) demonstrate a clearance of NA progressing distally in the nerve below the crush with time after crushing This decrease of 45 % is most probably caused by a further transport distally of this NA fraction The distal transport occurred in spite of the interrupted connection with the cell bodies in agreement with earlier reports on a redistribution of NA in double ligated sciatic nerves (Dahlström 1967 b) The fast transport of amine granules distal to a crush is also in agreement with studies on the intra axonal transport of labelled proteins distal to a crush (Ochs and Ranish 1969)

Some information about the rate of the fast transport distal to the crush can be obtained from the disappearance pattern *1 h after crushing* the NA decrease was observed at least within one 5 mm section of the nerve (segment C Fig 1) The rate of transport therefore appears to be at least 5 mm/h

2 h after crushing the NA clearance was observed within at least 2 segments (B + C) with a tendency to decrease also in segment D The distance may thus be more than 10 mm and



Fig 2 Fluorescence microphotograph of an air dried stretch preparation of the connective tissue sheath of a normal rat sciatic nerve Small arteries with strongly fluorescent adrenergic nerve terminals can be observed (—→) 160

the time for active transport was about 2 h The rate of distal transport thus appeared to be between 5 and 10 mm/h

3 h after crushing the NA was decreased within a distance of at least 25 mm from the crush (segments B E) The time for active transport was about 3 h which will give a transport rate of about 8 mm/h

6 and 12 h after crushing the decrease in NA was observed in all investigated segments indicating that the transportable NA had been transported into segments further distally than the most distal segments observed in the nerve The distance for transport is thus longer than 30 mm and the rate probably faster than 5 mm/h

These results indicate that the rate of NA transport distal to a crush is faster than 5 mm/h but lower than 10 mm/h and probably around 8 mm/h Shorter nerve segments would have to be used to obtain more exact values This would be difficult for practical reasons since then more than 20 nerve parts would have to be pooled with the present method

The maximal decrease of NA in the nerve segments distal to the crush was found to be 45 of the normal content (Fig 1) This was obviously the fraction of NA which had been transported further down the axons after the crush indicating that in the rat sciatic

nerve only 45% of the NA content is transportable. The remaining 55% appears to be either stationary or very slowly migrating.

The location of the stationary or slowly migrating fraction of NA was studied. In the connective tissue sheath, assayed separately for NA content (Table II), a large amount of NA was present. Histochemical studies of air-dried spread preparations of the sheath revealed the presence of many blood vessels with NA containing nerve terminals (Fig. 2). Also in the core preparation, consisting mainly of different kinds of axons, some adrenergically innervated blood vessels were seen in the endo- and perineural connective tissue. It is therefore likely that at least most of the stationary NA is located within adrenergic vasomotor nerve terminals. It cannot be excluded that a small part of the stationary NA may be located inside the adrenergic non-terminal axons in a form which does not permit rapid intra-axonal transport.

In the sheath preparation (Table II) a small, non-significant decrease of about 20% was observed. Since non-terminal axons were observed histochemically in the sheath preparation, a distally directed transport in these axons may explain the decrease in NA. Likewise, a NA decrease of 45% would be expected to occur in the core preparation 6 h after crushing if this preparation contained all the adrenergic axons and no nerve terminals. A decrease of only 37% was, however, observed, which may be due to the presence of adrenergic nerve terminals (not losing NA after crushing) in vessels around the axon bundles. The total decrease in the dissected 2 cm segments after crushing (sheath and core taken together) was 35%, *i.e.* 10% less than what would be expected from the data in Fig. 1. This may be due to losses of axonal NA from the core during the preparation procedure.

In cholinergic nerves a rapid transport of acetylcholine (ACh) has been demonstrated (Häggendal *et al.* 1971, 1973, Dahlström *et al.* 1974). In these nerves (sciatic motor nerve and autonomic preganglionic nerve) the fraction of ACh which is rapidly transportable distal to a crush is only about 20 and 5% of normal, respectively. In these nerves the stationary fraction is probably not located in vasomotor nerve terminals, since the connective tissue sheath (carrying most of the blood vessels) contained no detectable amounts of ACh (Dahlström *et al.* 1974).

The fact that only a part of the NA content in the sciatic nerve is transportable is of great importance for calculations on the rate of NA transport using values for the NA accumulation proximal to a crush. Earlier a rate of 5 mm/h was calculated based on the assumption that all of the NA was transportable (Dahlström and Häggendal 1966). With the present information that only 45% is mobile, and using the new information on the NA loading of the granules (see accompanying paper by Dahlström *et al.* 1975), a rate of 9 mm/h has now been calculated, which is in good agreement with the figure of 8 mm/h for transport *distal* to a crush obtained in this study. This clearly demonstrates the importance of estimating the *transportable fraction* of any substance investigated for intra-axonal transport by accumulations proximal to a crush or ligation (*cf.* Häggendal *et al.* 1973, Dahlström 1973).

Dopamine β-hydroxylase (DβH), an enzyme bound to the amine storage granules, has been studied with respect to intra-axonal transport and rapidly transportable fraction. About 30% of the normal DβH content in the sciatic nerve (Wooten and Coyle 1973) and

less than 50% (Haggendal *et al* 1974) was found to be rapidly mobile Based on these values and on accumulation data above a nerve crush a rate of transport of 6–8 mm/h was calculated for DβH (Wooten and Coyle 1973) This is in excellent agreement with the present data for transport of NA storage granules using NA as a marker instead of DβH

Support has been obtained from the Swedish Medical Research Council (grants Nos 14X 166 14X 2 07 and 04P-4173) from Magnus Bergvall s Foundation and from Wilhelm & Martina Lundgren s Foundation The technical assistance of Mrs Elizabeth Ljung and Miss Mildred Pettersson is gratefully acknowledged

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Acta physiol scand 1975 94 393–397

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Synaptosomes Containing Large Agranular Vesicles Isolated from Developing Rat Brain

By

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Received 16 January 1975

Abstract

KANERVA L A HERVONEN and A H TISSARI *Synaptosomes containing large agranular vesicles isolated from developing rat brain* Acta physiol scand 1975 *94* 393–397

Using the subcellular fractionation technique the fine structure of the isolated nerve endings (synaptosomes) from the hemispheres and brain stem of the 1-day old and adult rat was examined In the synaptosomal fractions of the brain of 1-day old rats we observed a new type of nerve endings containing predominantly large agranular ves cles about 1 000 Å in d ameter After incubation with α methylnoradrenaline or 5 hydroxydopamine these vesicles remained agranular It is assumed that the new type of large ves cles represent developing synaptic vesicles

The subcellular fractionation technique (De Robertis *et al* 1961 Gray and Whittaker 1962) gives a unique possibility of studying large numbers of nerve endings with electron micros copy and of observing morphologically different types of nerve endings within a reasonable number of tissue sections This technique seems especially suitable for studies of immature nervous tissue which has a low frequency of nerve terminals at different degrees of matura tion (for ref see Hervonen *et al* 1974 Kanerva *et al* 1974 a) We have used the subfraction ation technique to study by electron microscopic and biochemical means maturation of nerve ending during ontogeny in rat and human brain (Hervonen *et al* 1974 Kanerva *et al* 1974 b Tissari 1973 1975) Information on the ultrastructure of synaptosomes from developing brain could also contribute to an understanding of the origin and significance of different types of synaptic vesicles and other intraneuronal structures We have observed in the brain of 1-day old rats a new type of synaptosomes containing predominantly large agranular vesicles described in the present paper

Material and Methods

Adult and 1-day-old Sprague D wl y rats w re used n some experiments an mals pretre t d with a monoam ne oxidase nh b tor pargyl ne HCl (Eutonyl* 100 mg kg i p 1 h before k ll ng) were included S bfractions we e prepared from the hem spheres nd bra n stem as previously descr bed (see Hervonen

et al 1974) The crude mitochondrial fraction (P_2) was resuspended in 0 32 M sucrose and layered over a discontinuous sucrose gradient consisting of layers of 1 4 1 2 1 0 and 0 8 M sucrose The gradient was centrifuged and the layers were collected by aspiration diluted with water to sucrose concentration 0 32 M for A and B and to 0 55 M for C D and E Subfractions were centrifuged and the pellets suspended in Krebs bicarbonate solution pH 7 4 (KRB) In some experiments fraction P_2 prepared from the striatum and hypothalamus of the adults and from the hemispheres diencephalon mesencephalon and pons medulla oblongata of the 1 day old rats was used and suspended directly in KRB Aliquots were removed at 0 C, suspensions preincubated at 37 C and 95 O_2–5 CO_2 in a water bath shaker for 8 min and L-α methyl noradrenaline HCl (α methyl NA Corbasil®) or 5 hydroxydopamine HCl (5 OH DA H 83/35) added to final concentrations of 10 or 100 µg/ml Ascorbic acid to 0 2 mg/ml was also added to some samples Incubation was continued for a further 15 and 30 min aliquots were removed and cooled in an ice bath Controls without substrate were incubated for the same period Samples were fixed either as a suspension or pellet in $KMnO_4$ (final concentration 3 in 0 1 M sodium phosphate buffer pH 7 0 for 30 to 60 min) or 2 5 5 glutaraldehyde (in 0 1 M sodium phosphate buffer pH 7 4 for 30 to 180 min) Pellets were sedimented by centrifugation at 10 000 g for 15 min and washed after fixation in the buffer for 12 h The glutaraldehyde fixed specimens were postfixed in phosphate buffered 1 OsO_4 at pH 7 4 for 1 to 2 h Then the specimens were dehydrated and embedded in Epon Araldite The $KMnO_4$ fixed sections were viewed and photographed unstained while the glutaraldehyde fixed specimens were stained on grids with lead citrate and uranyl acetate A Philips EM 300 electron microscope operated at 40 to 60 kV was used

Results and Discussion

Most of the synaptosomes of the adult and 1 day-old rats contained mainly small agranular spherical vesicles and a few large granular and agranular vesicles after fixation in glutaraldehyde followed by osmium tetroxide or in $KMnO_4$ After incubation with α methyl NA or 5 OH–DA and fixation in $KMnO_4$ small granular vesicles were found in less than 1% of the synaptosomes of the fraction P_2 or subfractions of the adult and even less frequently in those of the 1-day-old rats A third type were the synaptosomes which contained predominantly large agranular vesicles about 1 000 Å in diameter and no or a few small agranular vesicles after $KMnO_4$ fixation and were found in the brain of 1 day-old rats (Fig 1) These synaptosomes occurred in all subfractions which contain nerve endings in 1 day-old rats namely A B C and D After incubation with α methyl NA or 5 OH–DA these vesicles remained agranular After $KMnO_4$ fixation the internal structure of the large agranular vesicles consisted of fine reticulum the density of which varied only slightly from vesicle to vesicle and was slightly higher than that of the surrounding axoplasm Some of these vesicles appeared elongated (Fig 1) The synaptosomes with the large agranular vesicles also contained the normal organelles of nerve endings small mitochondria occasional neurotubuli and probably a greater abundance of smooth endoplasmic reticulum than the other types of synaptosomes In adult rats synaptosomes containing an abundance of large agranular vesicles could be detected less often

In the peripheral nervous system axons containing large opaque or agranular vesicles have been demonstrated and attributed to non-cholinergic (purinergic) inhibitory nerves (Robinson *et al* 1971 Burnstock 1972 Hervonen 1973) while the inhibitory nerves of the central nervous system have been connected with flattened small agranular vesicles (Uchizono 1965)

In studies of the ultrastructure of developing adrenergic innervation of rat pineal gland Machado (1971) found that the proportion of small granular vesicles increases with age and that of small agranular vesicles decreases while the absolute number of large granular vesicles



Fig. 1 Synaptosomes of crude m tochondrial fraction (P) prepared from the hemisphere of one-day-old rat (the animals were pretreated with pargyline and the fraction was incubated with 10 μg ml 5-OH DA for 30 m n) One synaptosome (a) conta ns a moderate number of small agranular synaptic esicles a few large agranular esicles (thin arrow) and a mitochondrion (m) The other nerve end ng (b) contains many large agranular vesicles some of which appear flattened (thick arrow) The agranular ves cles ha e an inter nal structure consisting of f ne reticulum The nerve end ng also contains many arrays of endoplasm c re ticulum (asterisc) Fixat on as a suspens on n 3 KMnO 66 000

decreases slightly He concluded that large granular vesicles might have some functional significance in the early development of adrenergic fiber but might not be young forms of small granular vesicles as suggested by Geffen and Ostberg (1969) Vesicles may be formed from smooth endoplasmic reticulum (SER) and the dense material may appear in the SER before and after (large granular vesicles) or after (small granular vesicles) the vesicles have pinched off from the reticulum (Machado 1971) Almost all components of the neuron have been proposed as the origin of the synaptic vesicles mitochondria nerve cell membrane complex vesicles large granular vesicles the Golgi apparatus and the smooth endoplasmic reticulum (for ref see Kanerva *et al* 1974 a) The endoplasmic reticulum has also been suggested as a site of amine store (Tranzer and Thoenen 1967 Eranko 1972 Tranzer 1973) In ligated rat sciatic nerve proximal to the ligature local morphogenesis involves rapid outgrowth of endoplasmic reticulum and appearance of empty vesicular and tubular profiles (Taxi and Sotelo 1973) In the present study synaptosomes of the 1-day-old rat containing large agranular vesicles had also smooth endoplasmic reticulum abundantly and elongated vesicles, possibly newly formed ones Interestingly in a phylogenetically old group the cephalopods, large agranular vesicles have been found in the synaptosomes of the adult octopus vulgaris (Jones 1967)

It is assumed that the new type of large vesicles described in the present paper represent non-degenerative developing synaptic vesicles Their transmitter nature is not known although the possibility of monoaminergic origin does come to mind Further study on ultrastructure of developing synaptosomes is in progress A grant from the Paulo Foundation is greatfully acknowledged

This work was finished while one of us (LK) was on a Public Health Service International Re earch Fellowship (Number I F05 TW0 053 01) at the University of California School of Medicine Department of Human Anatomy Davis California 95616 USA

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Acta physiol scand 1975 94 398–400
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Group II Excitation in Motoneurones and Double Sensory Innervation of Extensor Digitorum Brevis

By

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In a recent investigation (Lundberg *et al* 1975) of the effects of electrical stimulation of group II muscle afferents we noted that very large EPSPs are evoked in flexor motoneurones from the nerve to SPM (superficial peroneal muscles peroneus longus tertius and brevis) By contrast the effect from the nerve to DP (deep peroneal tibialis anterior and extensor digitorum brevis) was weak or entirely lacking The explanation may be either that secondary spindle afferents from the two groups of muscles differ with respect to their central actions or else that the effect from the SPM nerve is evoked from afferents of non spindle origin

As part of a further analysis we have now investigated separately the effect from the nerves to peroneus longus tertius and brevis Intracellular recording was made from knee flexor motoneurones It was a regular finding that group II volleys from peroneus tertius or longus evoked only small EPSPs and that virtually the entire SPM group II effect was produced from the peroneus brevis nerve However we noted that this nerve contains a small *extra branch* which does not go to peroneus brevis but continues along the dorsal surface of the peroneus brevis tendon to enter the foot on the dorsal side of the lateral malleolus Fig 1 B–D shows that a major part of the group II EPSP from the peroneus brevis nerve is not evoked from the proper branches to this muscle (C) but from the extra branch to the foot (D) Stimulation of this branch did not evoke any muscular contraction in the foot and we expected that its fibres originated entirely from extramuscular tissue However dissection of the course of this branch in the foot (5 cats) revealed that it passes into extensor digitorum brevis (EDB) and pulling the transected tendon gave a multifibre discharge which had all *the characteristics of a discharge in muscle spindle afferents*

EDB is known to be innervated via a nerve which runs with the DP nerve and courses distally along the inner surface of tibialis anterior On reaching the dorsum of the foot it divides into a lateral branch to skin and a medial branch which passes into EDB We have dissected the latter branch and compared its electroneurogram evoked by stimulation of the appropriate ventral and dorsal roots with that recorded in the extra branch to EDB

Fig 1 E F show that all α and -efferents to EDB are in the regular nerve Both branches contain group I and II afferents but the former predominate in the regular nerve and the

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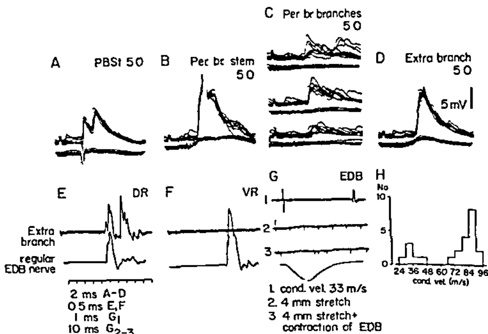


Fig 1 A–D upper traces intracellular recordings from a motoneurone to posterior biceps-semitendinosus (PBSt) lower traces from the dorsal root entry zone in L7 A Ia and group II EPSP evoked from the PBSt nerve B–D EPSPs from Per br stem and branches from it as indicated see text and observe also similarity in negative cord dorsum potential in B and D E–F recording from extra branch (dist. 23 4 cm) and regular nerve to EDB (dist. 25 9 cm) on stimulation of appropriate dorsal (DR) and ventral root (VR) as indicated Strength of stimulation in A F 5 0 times threshold G 1–3 single unit dorsal root filament recording from secondary EDB afferents running in the extra branch which was in intact connexion with EDB the EDB tendons were dissected free and connected to strain gauge and pulling device Contraction of EDB by stimulation of efferents in regular nerve H histogram of conduction velocities of spindle EDB afferents in the extra branch. See text. Spinal cats anesthetized with chloralose

latter in the extra branch The fastest group I afferents in the extra branch conduct at 90 m/s *vs* 105 in the regular nerve Single unit recordings from dorsal root filaments confirmed that spindle afferents from EDB primaries as well as secondaries course in this extra branch The graph in H gives the distribution of conduction velocities in spindle afferents found in a systematic analysis of all dorsal root filaments with axons in the extra branch (regular nerve cut) The arbitrary setting of 72 m/s as dividing line (*cf* Matthews 1972) gives 16 primary and 7 secondary afferents in the extra branch However the distribution in two separate groups in H rather suggests a general downwards displacement of conduction velocities If so all the fibres between 96–66 m/s may be primaries although a conduction velocity above 80 m/s usually is required in order to attribute with some confidence a spindle afferent to a primary ending (Matthews 1972) EDB has 56 muscle spindles (B... and Davey 1968) and if we assume that some secondaries were missed in the filament analysis then we arrive at the suggestion that the extra branch contains about 25 of the ... afferents from EDB They seem to originate mainly from the lateral head of EDB ...

the extra branch enters Spindle afferents from the two medial heads run in the regular nerve as evidenced by the mass discharge evoked by separate pulling of their tendons In summary our results show that there is a double nerve supply to EDB α and γ-efferents are entirely in the regular nerve to EDB while part of the spindle afferents run in a separate nerve the extra branch which joins the peroneus brevis nerve

The problem at hand is whether the large group II EPSP evoked in knee flexor motoneurones on electrical stimulation of the extra branch (D Fig 1) is evoked from secondary EDB afferents or from non-spindle afferents The dorsal root filament analysis revealed that the prominent group II peak in the extra branch (Fig 1 E) is contributed largely by non spindle afferents We found 61 group II fibres from receptors outside EDB most of which which were activated by strong deep pressure of the toes or the metatarsi but some from the skin of the lateral aspect of the foot Thus the ratio of secondary afferents to non-spindle group II afferents in the extra branch is about 1 10 The synaptic activity evoked via the extra branch on adequate stimulation of the foot was investigated with intracellular recording from knee flexor motoneurones Stretch of EDB gave only very weak excitatory synaptic noise while marked activity was produced by pressure of the toes or metatarsi Altogether the present findings strongly suggest that the large group II EPSP evoked in knee flexor motoneurones from the extra branch is mainly due to activation of afferents from extra muscular receptors in the foot

This work was supported by the Swedish Medical Research Council (Project No 94)

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Acta physiol scand 1975 94 401–422
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The Capillary Permeability of the *rete mirabile* of the Eel, *Anguilla vulgaris* L

By

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Received 9 January 1975

Abstract

STRAY PEDERSEN S and J B STEEN *The capillary permeability of the rete mirabile of the eel Anguilla vulgaris L* Acta physiol scand 1975 *94* 401–422

Direct measurements of transcapillary exchange during steady-state conditions have been performed in the *rete mirabile* of the eel The capillaries were found to be more than 30 times as permeable to THO (P_{THO} = 33.2 × 10^{-5} cm sec^{-1}) ethanol and antipyrine than to K^+ The mutual relationship of the permeabilities for K^+ Na^+ urea and sucrose were similar to that between the corresponding free diffusion coefficients in water The permeability characteristics did not change when the perfusate contained metabolic inhibitors Addition of 1 mM/l of albumin lead to a significant transcapillary osmotic flow NaCl urea and sucrose however did not cause osmotic flow Based on the quantitative values for permeability and volume flow the capillary pores should be about 1300 Å and have a frequency of 1 3 × 10 per cm^2 of the capillary surface occupying an area of about 0 1 ‰ of the total area The results obtained in the present investigation raise questions as to the validity of the Pappenheimer single pore theory for transcapillary transport as well as to the fine structure of the capillary pores As an alternative to Pappenheimer s theory a multi pathway model is proposed in order to explain transcapillary transport

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The capillary wall is made up of flattened endothelial cells and a basement membrane The width of the cell junctions (pore or slit diameter) varies considerably from one organ to another In skeletal muscles of mammals it is about 40 Å (Karnovsky 1968) In the brain capillaries the outer leaflets of the apposed cell membranes appear to be fused (= tight junctions) (Reese and Karnovsky 1967)

There is general agreement that the transport of gases water ions and small substances across the capillary barrier is a passive diffusion process (Kruhoffer 1946) Lipid-soluble substances diffuse mainly through the endothelial cells whereas water soluble substances pass through the capillary wall via the intercellular clefts

Pappenheimer *et al* (1951) and Pappenheimer (1953) presented an extensive and detailed model for the porous exchange They based their model on the results obtained from studies of the osmotic transients of different substances during perfusion experiments on cat hind limbs They found that the osmotic effect of molecules of varying sizes could be quantita

tively accounted for by assuming that 1) transport of both water and other substances occurred only through the pores and 2) the pores restricted diffusion progressively the closer the diameter of the substance was to that of the pore

Later research has lent both support and doubts as to the validity of this model Beck and Schultz (1972) established that mica sheets with uniform straight pores with diameter of 90 Å cause approximately the same molecular size dependant restriction to diffusion as postulated for capillaries by Pappenheimer *et al* (1951) Michel (1970) has shown that the permeability to Patent Blue and ions of the mesenterial capillaries of the frog is far greater at the clefts than across the cells But it has also been shown in the rabbit placenta (Faber and Hart 1967) and in the dog heart (Alvarez and Yudilevich 1967) that the capillaries are significantly more permeable to THO than should be expected if the slits were the only pathway for diffusion Crone (1963) was not able to demonstrate any diffusional restriction to sucrose and inulin in muscle capillaries whereas a considerable restriction to Cr EDTA and inulin was found by Trap-Jensen and Lassen (1971) In the capillaries of the dog heart Alvarez and Yudilevich (1969) found no restriction to smaller molecules whereas Schafer and Johnson (1964) and Vargas and Johnson (1967) found permeability values for sucrose and inulin strongly indicating a restricted diffusion

Capillaries are unfortunately not readily accessible for permeability studies In the investigations mentioned different methods have been employed, such as the indicatordilution method and other modifications of clearance procedures and the osmotic transient method All of these techniques give indirect data and can never be carried out under steady state conditions Also since the capillaries make up only a minor fraction of the tissue it might be questioned whether the permeabilities obtained from these studies actually reflect the properties of the capillary membrane proper or involve diffusional properties of extravascular tissue and fluid as well

The capillary bundle of the *rete mirabile* connected to the swimbladder of the eel appeared to us as an organ of choice for studies of capillary exchange Krogh (1929) called attention to the unique anatomy of the *rete* A single *rete* has a cross-sectional area of 8 mm and contains 58 000 arterial and 44 000 venous capillaries The capillary surface area in each of the capillary systems is as great as 53 cm These measurements have been confirmed by Stray Pedersen and Nicolaysen (1975)

Electron microscopical studies of the capillaries have been performed by Dorn (1961) and Fawcett (1963) and by Stray Pedersen and Nicolaysen (1975) The arterial capillaries have endothelial cells of variable thicknesses from 0 2–1 μm The average width of the junctions between the cells is 110 Å The endothelial cells of the venous capillaries are thinner about 0 15 μm and richly fenestrated The junctions are somewhat wider (average width 120 Å) Both types of the endothelial cells contained numerous (pinocytotic) micro-vesicles and several giant vacuoles The arterial capillaries may therefore be compared to the capillaries of skeletal and heart muscles of mammals whereas the venous ones are morphologically similar to those found in the intestinal mucosa of mammals

Perfusion of the *rete* with fluids of known compositions allowed direct measurements of the steady-state exchange of substances as well as of osmotic and hydrostatic flow between the arterial and venous capillaries On the basis of these experiments we were able to postulate

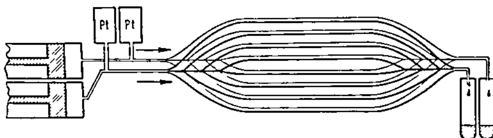


Fig 1 Schematic drawing of the experimental design (parallel perfusion) The perfusates one reference solution containing the test substances and one zero-solution were delivered to the *rete* from two syringes (left in figure) in a pump The input pressures were measured by pressure transducers (Pt) connected to the input cannulas by T tubes The outputs from the *rete* were collected in two tubes (right in figure) The outflow was measured by we ghing the tubes before and after the sampl ng. The concentrations of the substances to be studied were measured in the output samples as well as in the input solutions.

a physiological model of the capillary membrane in the *rete* and make a comparison between this and the capillary model generally accepted

Methods

Operation techn q e

The eel was anesthetized by submersion in a 0 05 solution of MS ...2 in water or made unconscious by a blow on the head The blood supply to the swimbladder was ligated and the organ removed The bladder was opened spread out and fastened by pins Polyethylene cannulas were inserted in the artery and vein on the proximal (heart) side of the *rete* (see Fig 1) and the organ was flushed with heparinized plasma containing papaverine in order to wash out the blood cells. Small glass tubes were inserted in an artery/ ve n pair on the bladder side of the *rete* and fastened by ophthalmic suturing materials The numerous side br nches from these ma n vessels were closed by special broad plated bulldog clamps

The input tubes of the *rete* were connected to a perfusion pump and output fluid was collected in test tubes placed below the preparation plate (Fig. 1)

Perf s n

Constant flow perfus on of the *r te* was carried out with Unita Perfusor Pumps (Braun–Melsungen) with an accuracy w thin 0.2 of the delivery

The perfusion pressure in both input cannulas were continuously recorded by connecting a side branch to a Statham P?3De transducer (Fig. 1) This served as a continuous control of the perfus on conditions. The main type of accidents occurring in these m cro-perfusions was that of tiny air emboli in the per fusates If these were not eliminated immediately the experiment was spoiled

The flow rate was normally adjusted to give a perfus on pressure of about 30–40 cm H_2O in the in put artery This pressure corresponds to the mean *in r o* pressure measured in the dorsal aorta of eels (Mott 1954) and to the blood pressure in the swimbladder artery (own measurements) At such conditions the perfusion pressure in the venous input most often was in the range of 10–15 cm H O

In most experiments the arterial and enous system of the *r te* was perfused in a parallel fashion using the vessels at the heart pole of the *ete* as input vessels. During parallel perfus on the risk for shunts and leakages to occur was less than with counter-current flow In some e periments however counter current perfus on was employed Samples of output were collected for 2–15 min depending on the flow rate The samples were we ghed and the outflow rate determ ned

Perf sates

Horse plasma was obtained by centrifugation of heparinized horse blood (25 I U ml of Heparin A L.) for 15 min at about 1000 g. The plasma was di ided in appropriate batches and frozen Prior to an experi ment a plasma b tch was thawed at room temperature and f ltered through one layer of f lter pape

TABLE I Relevant physico-chemical data of the test substances

Substance	MW	Molecular (ionic) radius (Å)	D_s ($\times 10^5$ cm sec^{-1})	Olive oil/water partition coefficients
(Water)	18	1 5	2 66 [1]	0 007 [6]
THO	20	1.5	2.44 [1]	do (?)
Na^+	23	0 95 (as nonhydrated)	1 48 as NaCl [?]	Insoluble
K^+	39	1 33 (as nonhydrated)	1 917 as KCl [?]	do
Ethanol	46	2.4[a]	1.4 [3]	0 0 2 [6]
Urea	60	2 64	1 38 [4]	0 00015 [6]
Antipyrine	188	4 1[b]	0 68	0 012 [6]
Sucrose	34	5 55	0.5? [4]	Insoluble
Dextran-75 000	60 000–90 000	60	0 036 [5]	do

[a] Calculated as Stokes-Einstein radius $r = RT/6\pi\eta DN$
[b] Calculated as equivalent sphere radius $r = (3M/4\pi\rho N)^{1/3}$
(M is the molecular weight, ρ is the density N is Avogadro s number R is the gas constant, T is the absolute temperature and η is the viscosity of the solvent)
Found by extrapolation from the diffusion coefficients of urea glucose and sucrose

References [1] Wang *et al* 1953 [ ] Handbook of Chemistry and Physics 1971 [3] Johnson and Babb 1956 [4] Longsworth 1954 [5] Personal communication from New England Nuclear [6] Collander and Bärlund 1933 [7] International Critical Tables 19 9

Eel plasma was used in a few expts Blood was collected from the ventral aorta of 3-4 eels heparinized centrifuged and filtered in the same way as for horse plasma.

Papaverine sulphate was added to the perfusates (0 1 mg ml blood) in order to achieve total paralysis of the vasculature (Stray Pedersen 1970) and thereby obtain stable perfusion conditions

In order to set up a gradient across the *rete* capillaries the substance under study was added to the arterial or to the venous perfusate To maintain iso-osmotic conditions the addition of test molecules to one perfusate was balanced by addition of equimolar amounts of NaCl to the other perfusate

Test substance

The capillary permeability of the following substances were studied

As isotopes

Na (as NaCl, Institutt for Atomenergi Kjeller Norway) K (as KCl do) antipyrine N Methyl-^{14}C (Catalogue number NEC 398 New England Nuclear Boston Mass) urea ^{14}C (NEC 108 NEN) sucrose-^{14}C (NEC 100 NEN) ethanol ^{14}C (NEC-079 NEN) dextran-carboxyl ^{14}C MW 60 000 90 000 (NEC 218B NEN), and THO (Institutt for Atomenergi Kjeller Norway)

As non-isotopes

K (as KCl), Na (as NaCl) Cl (as NaCl)

Relevant physical data of the tracers are listed in Table I

Chemical analyses

The concentration of K and Na were measured with an Eppendorf flame photometer The determination of K and Na was accurate to within at concentrations in the range of normal human plasma values. Cl was determined with an accuracy of ±1 on a chloridemeter (electrical titration) (Fel)

Radiochemical analyses

A 3 Channel Auto Gamma Spectrometer and Tri Carb Liquid Scintillation Spectrometer (Packard Instrument Co) were used for the measurements of radioactivity Precautions were taken in order to count

the isotopes separately To achieve high accuracy the activities in the reference solutions were adjusted when possible to give at least 10 000 c p m in the output samples As scint llation fluid was used Instagel (Packard Instrument Co)

Assumptions and Calculations

The capillary permeabil ties were computed from the data of flow and concentrations of the test molecules in the input and output solutions obtained during steady state cond tions of perfusion (steady state = the pressures the input and output flow and the concentrations in the in and output fluids being constant during the actual experimental period

The output concentrations result from at least three d fferent exchange processes

1 Bulk flow through arterio-venous shunts
2 Hydrodynamic flow caused by a transcapillary hydrostatic or osmotic pressure difference (filtration)
3 Diffusion

The analytical results must be corrected for the contribution from 1 and 2 before the data are used to calculate the diffusional permeability

We recognized transcap llary bulk flow (shunts) as a difference between the concentration decline in one retial circuit compared to the concentration increase in the other Quantitative estimate of shunts was based on the dextran 75 000 values In e periments showing minimal leakages to the exterior bulk flow could also be detected by comparing the rate of output flow with that of the input. By opt cal in spection we observed only one locat on for the shunts namely at the output (bladder pole) of the *rete* In most cases it was caused by incomplete closing of the numerous anastomoses between the cannulated vessels in the bladder wall

The quantitati e extent of filtration was detected as a higher output than input concentrat on of dextran 75 000 in the ci cuit of the *rete* having the highest pressure

D ivation of pe meab l ty eq ations

The mathematical formulas were derived from a single capillary model (Krogh 1919) The arterial and venous vascular systems of the *rete* were reduced to two coaxial cylinders of equal length the surface of the inner cyl nder representing the area available to capillary exchange (Fig 2 and 3) The necessary symbols used in the derivation are listed in Fig 3 Notice that during counter-current perfus on $W_A = -W_V$

The formulas are valid only when the test substance is incompletely exchanged i e when exchange is diffusion and not flow limited As the arterial blood passes from x to x+dx (Fig 3) an inf nit simal change in concentration of solute $dc_{A,x}$, will take place due to exchange of solute over area A_C dx Using Fick s 1st law of diffusion for this exchange and assuming that $C_{A,0} > C_{V,0}$ the mass balance equat on for the arterial system will be

$$C_{A,x} W_A - C_{A,x+dx} W_A = -W_A \, dc_{A,x} = P \, A_C \, dx(C_{A,x} - C_{V,x}) \tag{1}$$

The correspond ng equat on for the venous system is

$$C_{V,x} W_V - C_{V,x+dx} W_V = W_V \, dc_{V,x} = P \, A_C \, dx(C_{A,x} - C_{V,x}) \tag{2}$$

The difference $C_{A,x} - C_{V,x}$ is called ΔC_x, i e

$$\Delta C_x = C_{A,x} - C_{V,x} \tag{3}$$

Different ating this equat on with respect to dx gi es

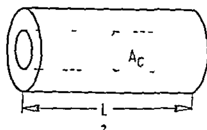
$$\frac{d(\Delta C_x)}{\Delta C_x} = \frac{dc_{A,x}}{dx} - \frac{dc_{V,x}}{dx} \tag{4}$$

Comb n ng Eqs. 1 2 and 4 and rear nging gi es

$$\frac{d(\Delta C_x)}{\Delta C_x} = -P \, A_C \left(\frac{1}{W_A} + \frac{1}{W_V}\right) dx \tag{5}$$

Integration gi es

$$\ln(\Delta C_x) = -PA_C\left(\frac{1}{W_A} + \frac{1}{W_V}\right)x + \text{const.} \tag{6}$$



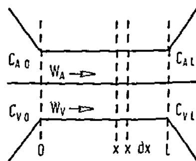


Fig. 2 Simplified model of the capillary systems in the *rete* The arterial and venous capillary systems are reduced to two coaxial cylindres of equal length (L) The circumferential area of the inner cylindre (A_C) represents the circumferential area available to capillary exchange

Fig 3 Simplified model of the capillary systems in the *rete* (Same as in Fig 2 the cylindres being sectioned longitudinally) List of symbols used in the figure and in the derivation of the permeability equations

C_{A0} = arterial pre rete concentration (e g mol cm^{-3})
C_{V0} = venous pre rete concentration (do)
C_{AL} = arterial post rete concentration (do)
C_{VL} = venous post rete concentration (do)
W_A = arterial flow (cm^3 sec^{-1})
W_V = venous flow (do)
L = length of the *rete* (cm)
A_C = circumferential area (cm) i e capillary surface area per unit path length
P = permeability (cm sec^{-1})
ΔC_0 = arterio-venous concentration difference pre rete
ΔC_L = arterio-venous concentration difference post rete
ΔC_A = difference between arterial pre rete and post rete concentrations
ΔC_V = difference between the venous pre rete and post rete concentrations.

When x = 0 $\Delta C_x = \Delta C_0$ and hence

$$\text{const} = \ln(\Delta C_0) \tag{7}$$

Which inserted in Eq 6 gives

$$\ln(\Delta C_x) = \ln(\Delta C_0) - PA_C\left(\frac{1}{W_A} + \frac{1}{W_V}\right)x$$

or

$$\Delta C_x = \Delta C_0 \exp\left[-PA_C\left(\frac{1}{W_A} + \frac{1}{W_V}\right)x\right] \tag{8}$$

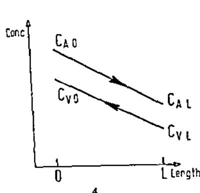
ΔC_x therefore changes exponentially as x goes from 0 to L.

If the arterial and venous system is perfused in counter-current fashion at equal flow ($W_A = W_V$) then $\Delta C_x = \Delta C_0$ which is to say that the arterio-venous concentration difference will be the same at all points along the *rete* and the arterial and venous concentrations will change linearly with capillary length (Fig 4) P can in the counter-current case at equal flow be calculated directly from Eqs 1 2 and 3 Setting $dc_{A\,x} = dc_{V\,x}$ and $W_A = W_V$ we get

$$P = \frac{W}{A_C L}\,\frac{\Delta C_A}{\Delta C_L} \quad \text{or} \quad \frac{W}{A_C L}\,\frac{\Delta C_V}{\Delta C_L} \quad \text{Counter-current flow} \tag{9}$$

Knowing the concentration difference at the output i e (ΔC_L) the permeability at parallel flow may be deduced from Eq 8

$$\Delta C_L = \Delta C_0 \exp\left[-PA_C L\left(\frac{1}{W_A} + \frac{1}{W_V}\right)\right] \tag{10}$$



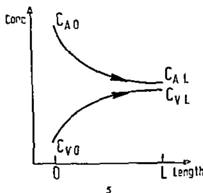


Fig. 4 Theoretical curves showing the change in arterial and venous concentrations in the *rete* as a function of the capillary length during counter-current perfusion at equal flow The symbols have the same meanings as in Fig 3

Fig 5 Theoretical curves showing the change in arterial and venous concentrations in the *rete* as a function of the capillary length during parallel perfusion at equal flow The symbols have the same meanings as in Fig 3

or

$$P = \frac{\ln \frac{\Delta C_o}{\Delta C_L}}{A_C L\left(\frac{1}{W_A} + \frac{1}{W_V}\right)} \tag{11}$$

If $W_A = W_V$ as was the case in our experiments

$$\boxed{P = \frac{W}{2A_C L} \ln \frac{\Delta C}{\Delta C_L}} \quad \text{Parallel flow} \tag{12}$$

Fig 5 shows the arterial and venous concentrations versus capillary length during parallel perfusion at equal flow

The diffusion coefficient (D) is equal to P Δx where Δx is the thickness of the barrier separating the 2 capillary systems P has the units of cm sec^{-1} D of cm sec⁻

The anatomical area (A) and the thickness (Δx) can be measured on fixed *retia* The actual size of the diffusional area and barrier thickness in the experiments however was seen to be highly dependent on the perfusion conditions Only in exceptional cases were the entire *rete* perfused The diffusional area of partially perfused *retia* could be estimated by inspection of organs which had been fixated after and an ink injection at the end of the experiment. This was done in some cases Microscopic inspection of cross sections indicated that perfused capillaries were predominantly found in bundles and not scattered throughout the *rete* Normally about 60% of the capillaries were perfused The true area was therefore less than the anatomical area whereas the anatomical distance between arterial and venous capillaries could correctly be used as arterio-venous diffusional distance

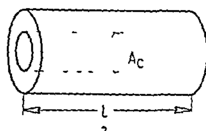
In the majority of the experiments the actual area available to transcapillary exchange was not measured We therefore calculated the permeability surface products (PS) = the capillary diffusion capacities (Renkin 1959 and 1968) for the test molecules

$$PS = W \frac{\Delta C_A}{\Delta C_L} = W \frac{\Delta C_V}{\Delta C_L} \tag{13}$$

in counter-current flow

$$PS = \frac{W}{2} \ln \frac{\Delta C}{\Delta C_L}$$

in parallel flow



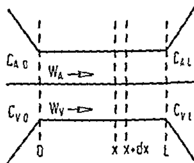


Fig 2 Simplified model of the capillary systems in the *rete* The arterial and venous capillary systems are reduced to two coaxial cylindres of equal length (L) The circumferential area of the inner cylindre (A_C) represents the circumferential area available to capillary exchange

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C_{VL} = venous post rete concentration (do)
W_A = arterial flow (cm^3 sec^{-1})
W_V = venous flow (do)
L = length of the *rete* (cm)
A_C = circumferential area (cm) i e capillary surface area per unit path length
P = permeability (cm sec^{-1})
ΔC_0 = arterio-venous concentration difference pre rete
ΔC_L = arterio-venous concentration difference post rete
ΔC_A = difference between arterial pre rete and post rete concentrations
ΔC_V = difference between the venous pre rete and post rete concentrations

When $x = 0$ $\Delta C_x = \Delta C_0$ and hence

$$\text{const} = \ln(\Delta C_0) \tag{7}$$

Which inserted in Eq 6 gives

$$\ln(\Delta C_x) = \ln(\Delta C_0) - PA_C\left(\frac{1}{W_A} + \frac{1}{W_V}\right)x$$

or

$$\Delta C_x = \Delta C_0 \exp\left[-PA_C\left(\frac{1}{W_A} + \frac{1}{W_V}\right)x\right] \tag{8}$$

ΔC_x therefore changes exponentially as x goes from 0 to L

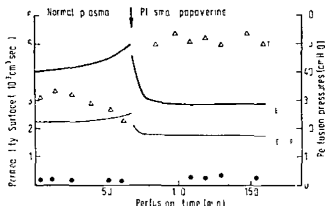
If the arterial and venous system is perfused in counter-current fashion at equal flow ($W_A = W_V$) then $\Delta C_x = \Delta C_0$, which is to say that the arterio-venous concentration difference will be the same at all points along the rete and the arterial and venous concentrations will change linearly with capillary length (Fig 4) P can in the counter-current case at equal flow be calculated directly from Eqs 1, 2 and 3 Setting $dc_{A,x} = dc_{V,x}$ and $W_A = W_V$ we get

$$P = \frac{W}{A_C L}\,\frac{\Delta C_A}{\Delta C_L} \quad \text{or} \quad \frac{W}{A_C L}\,\frac{\Delta C_V}{\Delta C_L} \qquad \text{Counter-current flow} \tag{9}$$

Knowing the concentration difference at the output side (ΔC_L) the permeability at parallel flow may be deduced from Eq 8

$$\Delta C_L = \Delta C_0 \exp\left[-PA_C L\left(\frac{1}{W_A} + \frac{1}{W_V}\right)\right] \tag{10}$$

Fig. 7 The effect of papaverine on the capillary exchange The curves and marks show the pressures and exchange obtained before and after addition of 10 mg/100 ml of papaverine to the perfusate Thick line represents the arterial pressure thin line the venous pressure $\Delta = PS_{THO}$ ● = PS_K



The low values for capillary exchange obtained when the flow was small was most probably due to scattered perfusion of the *rete* with concomitant reduced area and increased path of diffusion The increasing exchange at increasing flow is ascribed to a recruitment of new capillaries earlier unperfused At flow rates above a certain level the permeability ratios were constant which indicated that the diffusional path was now reduced to the minimal one (mainly capillary membranes) On the basis of a series of such flow-experiments it appeared that the achievement of optimal exchange conditions was more related to the perfusion pressure than to the flow Thus when the arterial perfusion pressure was 30 cm H O or higher the P_{THO}/P_{K^+} ratio was maximal and the distribution of indian ink in the capillaries was even

Effect of papaverine

The effect of papaverine on the capillary exchange was studied in 5 expts As can be seen from Fig 7 the pressure as well as the exchange was unstable during perfusion with normal plasma The exchange was low and the ratio between the permeabilities of THO and K^+ about 20 When papaverine-containing perfusates had been introduced the pressures fell abruptly and then remained constant The exchange of both substances however increased proportionally Similar results were obtained in the other experiments Papaverine therefore does not act on the permeability properties of the capillaries but contributes to more optimal perfusion conditions by paralyzing the smooth muscles of the retial vessels

Effect of metabolic inhibitors

The *rete* was perfused with normal perfusate until steady state conditions had prevailed for 30 min Then plasma containing 20 mM/l iodoacetate or/and 20 mM/l KCN was introduced This further perfusion lasted for at least one hour in one experiment for 2 1/2 h As test substances were used THO K^+ and ethanol

3 expts of this type were performed The results from a typical experiment are shown in Fig. 8 In none of these experiments did the introduction of KCN/iodoacetate lead to significant changes in perfusion pressure or capillary exchange The poisoned *retia* were examined histologically and no morphological changes could be observed

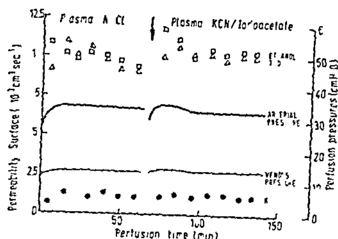


Fig 8 The effect of metabolic poisons on the capillary exchange The curves and marks represent the data for pressures and exchange obtained and after addition of 20 mM/l of KCN and 0 mM/l of iodoacetate to the perfusates during a perfusion Thick line represents the arterial pressure thin line the venous pressure □ = $PS_{Ethanol}$ Δ = PS_{THO}, O = PS_K+

Capillary filtration

The capillary hydraulic conductivity was estimated from the increase in the concentration of the non permeating substance dextran 75 000 in the high pressure circuit of the *rete*

The hydraulic conductivity (L_p) was calculated according to the equation

$$L_p = \frac{v_f}{\Delta P \cdot A} \tag{15}$$

in which v_f represents the volume filtered ($cm^3\ sec^{-1}$) ΔP the transcapillary hydrostatic pressure difference (cm H_2O) and A the capillary area (cm^2) L_p has the units of $cm^3\ cm^{-2}\ sec^{-1}\ cm\ H_2O^{-1}$

The capillary area (A) was not measured in these experiments but estimated on the basis of the exchange of THO (See Methods) ΔP was created by raising or lowering the output tubes and estimated from the equation

$$\Delta P = \frac{P_{A\,0} + P_{A\,L}}{2} - \frac{P_{V\,0} + P_{V\,L}}{2} \tag{16}$$

in which the subscripts 0 and L refer to the input and output side of the *rete*

Since it was not possible to establish a higher pressure in the venous system only capillary filtration in the arterio-venous direction could be measured

Four successful experiments of this type were performed The arterial output pressure was increased by 10-20 cm H_2O which lead to an increase in the transcapillary hydrostatic pressure of 7-16 cm H_2O followed by a net arterio-venous volume flow The size of this flow was about 2% of the input flow

Series of samples were taken during the filtration Using the area value estimated from the exchange obtained for THO the arterio-venous hydraulic conductivities fell within the range of 3 3-7 1 × 10^{-7} $cm^3\ cm^{-2}\ sec^{-1}\ cm\ H_2O^{-1}$ (Table II) The average value was 4 2 · 10^{-7} $cm^3\ cm^{-2}\ sec^{-1}\ cm\ H_2O^{-1}$ Since 1 cm H_2O difference of (osmotic) pressure is equal to 7.23 × 10^{-7} g/cm^3 difference of water concentration (the density of water = 1 0 g cm^{-3}) this value corresponds to an hydraulic permeability (P_f) of 0 58 cm sec^{-1}

TABLE II Net volume flow due to capillary filtration and the corresponding hydraulic conductivities obtained in a series of filtrations (4 expts)

Exp	Series of filtration	Input flow µl/min	Transcapillary hydrostatic pressure difference ΔP cm H_2O	Capillary filtration[a] v_f µl min	Capillary area[b] A cm^2	Capillary hydraulic conductivity L_p $cm^3 cm^{-2} sec^{-1} cm H_2O^{-1}$ × 10^7
I	1	[illegible]00	7	3 0	2[illegible]	3 3
			16	14 4		6 4
	3		16	8 6		4 1
	4		8	4 2		4 0
II	1	100	9	4 6	6	3 3
			9	4 9		3.5
III	1	375	11	7 5	27	4.2
	[illegible]		12	6 8		3 5
IV	1	100	8	3 4	[illegible]1	3 4
	2		13	5 9		3 6
	3		14	12 5		7 1

[a] Mean value of at least 3 measurements
[b] Estimated from the capillary exchange of THO

In no experiment did the concentration of K^+ urea or sucrose increase in the arterial system during a capillary filtration

The osmotic effect of albumin

An arterio-venous hydrostatic pressure difference of about 10 cm H_2O lead to a measurable rate of capillary filtration in the *rete* We would therefore expect a measurable osmotic flow of water when similar differences in osmotic pressure were imposed upon the system

The osmotic effect of albumin (bovine albumin VI Sigma) was studied in 2 expts The behaviour of dextran 75 000 during filtration suggests that the retial capillaries are virtually impermeable to albumin (MW 60 000) According to the empirical equation describing the osmotic pressure—concentration curves for albumin solutions (Landis and Pappenheimer 1963) an addition of 6 g/100 ml (1 mM/l) of albumin to one of the perfusates would create an osmotic pressure difference of about 35 cm H O The albumin-containing perfusate served alternatively as arterial or venous input As test substances were used THO K sucrose and dextran 75 000 These substances were added either to the albumine perfusate or to the other

The ratio between the viscosity of albumin plasma and that of normal plasma was found to be 1 43 by measurements of the perfusion pressures of these solutions through narrow polyethylene catheters The introduction of albumin was consequently followed by an increase of the perfusion pressure This pressure increase could be observed in both systems especially when albumin was added to the venous perfusate

Similar to the measurements of capillary filtration the osmotic flow was estimated from the data of dextran 75 000 and/or the output flow In order to calculate the osmotic conductivity (L_{pD}) the following equation was used

$$P_{osm} = \frac{V_{osm}}{(\Delta\pi - \Delta P) \ A} \tag{17}$$

in which v_{osm} represents the osmotic flow (cm^3 sec^{-1}) $\Delta\pi$ the transcapillary osmotic pressure difference (cm H_2O) estimated according to van t Hoff s law assuming $\sigma = 1$ and ΔP the transcapillary hydrostatic pressure difference calculated from Eq 16 (cm H_2O) The capillary area (A) was estimated from the exchange of THO obtained in the experiments L_{pD} has the same units as the capillary hydraulic conductivity (cm^3 cm^{-2} sec^{-1} cm H_2O^{-1})

Two experiments including 6 measurements of the osmotic flow were performed The osmotic conductivities were between $1\ 7–5\ 8 \times 10^{-7}$ cm^3 cm^{-2} sec^{-1} cm H_2O^{-1} with an average of $2\ 4 \times 10^{-7}$ cm^3 cm^{-2} sec^{-1} cm H_2O^{-1} This corresponds to a P_{osm} of 0 33 cm sec^{-1} The osmotic permeability in the arterio venous direction did not differ significantly from that obtained in the opposite direction K^+ and sucrose were not concentrated as a consequence of the osmotic flow

The osmotic effect of NaCl urea and sucrose

Since the *rete* capillaries are 50–100 times less permeable to NaCl urea and sucrose than to water one would expect that these substances would show osmotic activity In three experiments 25 mM/l of NaCl urea or sucrose was added to the arterial or the venous perfusate Assuming $\sigma = 1$ for these molecules this would give a transcapillary osmotic pressure difference of several hundred cm H_2O

2 expts of this type were performed THO K urea sucrose and dextran 75 000 served as test molecules In these experiments the concentrations of Na and Cl were also measured One set of experimental data obtained is shown in Table III

As may be seen from the table the addition of sucrose NaCl or urea was not followed by any significant changes in flow perfusion pressure or capillary exchange The increase in the arterial perfusion pressure was seen to take place continuously and was not related to the onset of the different perfusion The gain in venous outflow throughout the experiment was caused by an arterio-venous shunt on the output side as indicated from the data of sucrose and K

In the second experiment these substances were added to the zero-perfusate (containing no test-substances) which was used as venous input During none of the perfusions dextran 75 000 sucrose urea Na Cl or K was seen to accumulate in the arterial output thus clearly indicating that these substances were osmotically inactive to the capillary membrane

The tracer permeability of the rete capillaries

The capillary exchange varied considerably from one preparation to another These variations were obviously related to differences in the actual capillary area between the preparations This again could partly be due to differences in the perfusion conditions but also to the variations of the sizes of the *retia* employed

The area available to capillary exchange was estimated by the indian ink method on four *retia* which as judged from our criteria yielded optimal experimental conditions Table IV lists the capillary area the THO and the K permeability constants obtained in these organs The average value of $P_{THO} = 33\ 2 \times 10^{-5}$ cm sec^{-1} therefore represents the best approximation to the true permeability of the retial capillaries

In about 90 other perfusion experiments the capillary area was not measured The capillary

TABLE III The osmotic effects of NaCl urea and sucrose (Data from 1 expt) Input flow 0 ... ml min The table lists the data obtained in an experiment consisting of a series of plasma perfusions in which isotonic NaCl was continuously added to the venous perfusate whereas either isotonic NaCl 25 mM/l sucrose .5 mM/l NaCl or 25 mM/l urea was added to the arterial perfusate Abbreviations Inp A = arterial input Inp V = venous input Outp A = arterial output Outp V = venous output.

Perfusions	Input pressures (cm H_2O)	Flow (in of input flow)	Na^+ mE/l	Cl^- mE/l	THO c p m ($\times 10^{-3}$)	K^+ mE/l	Sucrose activity c p m ($\times 10^{-3}$)
I							
Inp A Isotonic NaCl	41	100	1??	10	?60	6 87	4 9
Inp V Isotonic NaCl	21	100	131	106	0	2 91	0 ?
Outp A		91 2	1??	101	146	6 78	4 7
Outp V		104 8	1 8	105	118	3 18	2 4
II							
Inp A ?5 mM/l sucrose	4?	100	11	89	261	6 59	4 5
Inp V isotonic NaCl	19	100	131	106	0	? 91	0 2
Outp A		91 6	113	91	151	6 48	4 0
Outp V		104 6	127	104	117	3 18	? 4
III							
Inp A 25 mM/l NaCl	44	100	148	130	261	6 59	4? 6
Inp V isotonic NaCl	19	100	131	106	0	91	0 ?
Outp A		91 0	141	1?3	154	6 50	42 4
Outp V		104 7	129	108	118	3 14	? ?
IV							
Inp A 25 mM/l urea	47	100	110	90	?58	6 60	42 0
Inp V isotonic NaCl	19	100	131	106	0	? 91	0 2
Outp A		91 5	113	9	152	6 48	41 4
Outp V		104 7	1 9	105	117	3 15	? 3
V							
Inp A isotonic NaCl	48	100	123	102	60	6 88	43 2
Inp V isotonic NaCl	19	100	131	106	0	2 91	0 2
Outp A		90 2	1?4	103	160	6 80	42 9
Outp V		104 6	131	105	12	3 18	? 5

diffusion capacities (or PS products) of the different test molecules were therefore related to that of the reference molecules THO and K and present in Table V as permeability ratios

In 14 of these expts there was no evidence of capillary filtration and the volume flow due to shunts or leakages was less than 5 of the input flow (ideal experiments) As demonstrated in Table V the permeability ratios P_{THO}/P_{K^+} were uniform and similar to those from the four experiments mentioned above

The remaining 74 expts suffered from various shortcomings and showed lower exchange ratios However as seen from Table V the general permeability pattern was not essentially different These experiments also include all of counter-current perfusions and the 3 expts with eel plasma as perfusate

The transport of K was found to be similar to that of (cold) K It was of no significance to the capillary exchange whether the test substances were added to the arterial or to the venous perfusate

TABLE VII Hydraulic (osmotic) permeabilities (P_f) tracer permeabilities of water (P_W) and equivalent pore radii (r_p) of different biological membranes

Membrane	P_f (10⁸ cm sec⁻¹)	P_W (× 10⁸ cm sec⁻¹)	P_f/P_W	r_p[a] (Å)	References
Eel rete capillaries	58 000	36[c]	1610[e]	167[f]	Present study
Cat skeletal muscle capillaries	4 00[b]	28[d]	150	48	[1]
Rat kidney proximal tubules	1 000	560	38	?4	[ ]
Toad bladder (with vasopressin)	1 880	16	118	40	[3]
Frog, ovarian egg	891	12 8	70	30	[4]
Human erythrocyte	1 ?70	530	?4	3 5	[5]

[a] Computed according to the relation quoted below (Eq 18)
[b] Calculated on the assumption of a capillary surface area of 7 000 cm²/100 g muscle
[c] Extrapolated from the tracer permeability of THO
[d] Extrapolated from (A_p/x) of NaCl
(η = viscosity of water D = free diffusion coefficient of water
$\bar{V}$ = molar volume of water R = gas constant T = absolute temperature)
Estimated on data from simultaneous measurements
[f] This value does not represent the intercellular pore radius (see text)

References [1] Landis and Pappenheimer 1953 [2] Persson and Ulfendahl 1970 [3] Paganelli and Solomon 1957 [4] Prescott and Zeuthen 1953 [5] Sjolin 1954

These calculations were performed as follows

If we assume the capillary pores to be much wider than the radius of the water molecule the pore radius (r_p) may be obtained from the following relation (Paganelli and Solomon 1957)

Eq 18 $$r_p^2 \quad (P_f/P_W \quad 1) \quad (8 n_W D_W \bar{V}_W / RT)$$

in which n_W represents the viscosity D_W the free diffusion coefficient $\bar{V}_W$ the molar volume of water R the gas constant and T the absolute temperature At ?3 C the factor $8 n_W D_W \bar{V}_W/RT$ = 14 3 10 cm²

The tracer permeability of water through the pores was obtained by extrapolation from P_X measured during the filtration experiments where L_p was estimated (Table II)

The area and the frequency of pores were calculated under the assumption of a functional capillary barrier of the rete constituting 50 of the anatomical surface and having a thickness of 1 6 μm (Stray Pedersen and Nicolaysen 1975)

We are aware of the questionable applicability of Poiseuille s law to flow through tortuous fibrous channels but also aware of the fact that laminar flow is less energy requiring than turbulent This method gives therefore the minimum pore size that can explain a flow of the magnitude measured at the pressure differences employed

We are therefore confronted with the following contradiction The hydrodynamic flow measured through the degree of accumulation of a 100 Å molecule (dextran 75 000) yields a pore diameter 13 times larger We suggest that the *in vivo* intercellular junction is about 1500 Å in diameter but filled with fibrous intercellular material This material will contribute little resistance to the hydrodynamic flow but constitute an effective molecular sieve

An immediate objection to this model is that intercellular junctions of this size have

never been observed We notice however that cell junctions have never been observed *in vivo* but only after fixation This procedure may well change the ultrastructure of junctions as much as freezing changes the structure of water Furthermore capillary beds which are made readily permeable to dextran 75 000 by removal of Ca^{++} with EDTA show no changes in the junctional structure (Stray Pedersen 1975) This has also been reported for the rabbit lung showing fulminant edema due to treatment with EDTA (Hovig *et al* 1971) The junctions of the *rete* still appear as channels no wider than 100–120 Å though significantly permeable to a 100 Å molecule

Admittedly our perfusion experiments did not allow precise estimations of the hydraulic conductivity (or P_f) The values obtained varied from experiment to experiment We therefore suggest the P_f-value quoted in Table VII to be accurate by a factor of 2 but comparable to the P_f of intestinal mucosa capillaries of rats (Folkow *et al* 1963)

Our high P_f-values could be due to widening of the pores as a consequence of distention of the capillary wall when the pressure was increased The presence of such stretched pore phenomenon in capillaries has been discussed but it seems doubtful if this phenomenon is present *in vivo* (Zweifach and Intaglietta 1968) In our experiments the capillary exchange of the test molecules seemed to remain unchanged during a filtration or osmotic flow The possibility might exist however that *some* pores could be substantially widened This would predominantly affect the size of the hydrodynamic flow since this depends on the fourth power of the pore radius whereas diffusional flow is related to the second power of the radius

The *rete* permeability constants and consequently also the diffusion constants of lipid insoluble molecules—except for THO—seem to be considerably smaller than those reported from other capillary beds (Table VI) However we have to consider that other workers may have great difficulties in estimating the actual capillary area as well as the diffusion distance in the organ investigated Pappenheimer *et al* (1951) assumed a capillary area of 7000 cm per 100 g of muscle and a wall thickness of 1 μ in order to calculate the permeability and diffusion constants in skeletal muscle capillaries In the *rete* the diffusional path length may vary tremendously from 0 5 μm up to several μm with an average of about 2 μm As judged from cross-sections of the *rete* it would be reasonable to suggest that far the greatest fraction of transcapillary transport would take place across an arterio-venous barrier with an average thickness of 1 6 μm constituting about 50 of the circumference of the capillaries (Stray Pedersen and Nicolaysen 1975) If we consider this barrier to represent the functional arterio-venous membrane then the retial capillary permeability (and diffusion constant) to for instance sucrose would be very similar to that obtained by Crone (1963) in the skeletal muscles of dog and by Alvarez and Yudilevich (1969) in the dog heart (Table VI)

From the direct observations of transcapillary transport as for instance reported by Karnovsky (1967) Schneeberger Keeley and Karnovsky (1968) and Michel (1970) there seems to be no doubt that the intercellular junctions serve as transport channels to hydrophilic molecules However the discrepancy between the pore dimensions calculated on the basis of permeability studies and those actually observed on electron micrographs (present study Faber and Stearns 1969 Boyd *et al* 1969 Faber *et al* 1971 Normand *et al* 1971

makes it tempting to suggest the existence of alternative transport pathways to these molecules In the dog brain it has been demonstrated that transcapillary transport of sugar molecules most probably takes place through the endothelial cells by facilitated diffusion mechanisms (Crone and Thompson 1973) Bruns and Palade (1968) have demonstrated that macromolecules such as ferritin (diam ~110 Å) are transported via the pinocytotic vesicles From kinetic studies it seems to be established that the vesicular system at least could represent the large pore system" of capillaries (Shea *et al* 1970) The presence of giant vacuoles and cytoplasmic extrusions (microvilli) in the *rete* endothelial cells (Stray Pedersen and Nicolaysen 1975) may indicate that transcapillary vacuolar transport may also occur in these capillaries Vesicular or vacuolar transport are generally assumed to take place by Brownian movements which are obviously too slow to account for the rapid exchange of for instance the ions As suggested by Bruns and Palade (1968) however the possibility might exist that vesicles may become fused and thereby form continuous chains through the endothelial cells In the *rete* small chains of 2–3 intercommunicating vesicles represented a rather common finding (Stray Pedersen and Nicolaysen 1975) Even though continuous chains in the luminal abluminal axis were never observed it can not be excluded that such chains may exist *in vivo*

It might also be that the vesicles (and vacuoles?) which appear as isolated structures on electron micrographs in fact represent sections of tortuous channels or tubules which are continuous throughout the cell This hypothesis has recently been proposed by Bendayan *et al* (1974) on the basis of ultrastructural studies of the retial endothelial cells According to these investigators the alternate dilatations and constrictions of the tubules observed on the micrographs may indicate the tubules to exhibit peristaltic activity A transcapillary transport through a system of contractile tubules may be considerably more rapid and require less metabolic energy than transport via pinocytotic vesicles The diameter of the dilated parts of the tubules was found to be as great as 1 300 Å which conforms precisely to the value for the dimension of the retial capillary pore calculated in the present investigation

Finally we would like to offer an alternative explanation to the experimental results of Pappenheimer *et al* (1951) which founded the single pore theory We believe that when plasma is made hyperosmotic with *e g* NaCl an osmotic dehydration of the cells is induced The simultaneously induced increase in venous pressure (in order to achieve isogravimetric conditions) will cause flow of plasma filtrate out through the junctions and into the extravascular space With time NaCl-diffusion into the cells will establish a new equilibrium When the plasma is made hyperosmotic by *e g* inulin the osmotic effect would be both across the endothelial cell membranes and across the junctions We shall be aware however that the osmotic behaviour of membranes with more than one population of pores available to the diffusion of water is poorly understood

One of us (S S-P) has been a Research Fellow of the Norwegian Research Council for Science and the Humanities Financial support from this Council and from the Nansen Foundation is gratefully acknowledged. Thanks are also due to Miss N Brewer and Mrs G Åkervik for skilful technical assistance

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The Effect of Ca^{++}, Mg^{++} and H^{+} on the Capillary Permeability of the *rete mirabile* of the Eel, *Anguilla vulgaris* L

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Received 9 January 1975

Abstract

STRAY PEDERSEN S *The effect of Ca^{++} Mg^{++} and H^{+} on the capillary permeability in the rete mirabile of the eel Anguilla vulgaris* L Acta physiol scand 1975 *94* 423–441

The effects of EDTA and of varying pH on the capillary permeability in the *r te mi ab le* have been investigated EDTA had to be added to the perfusate in excess to the total content of Ca^{++} and Mg^{++} in plasma in order to elicit changes in the permeability When exposed to sufficient amounts of EDTA the capillary permeability to hydrophilic molecules increased several fold whereas the permeability to THO and lipid-soluble molecules remained unchanged The increase in the permeability to high molecular dextrans was delayed When the EDTA-effect was maximal the mutual relat onship between the perme abilit es of THO and the lipid insoluble molecules became close to the ratio between the corresponding free diffus on coefficients in water During exposure to EDTA the content of K^{+} and Na of the *ete* tissue (mostly endothel al cells) became very similar to that of plasma Similar effects were obtained when the *rete* was perfused with plasma having a pH below 4 or abov 9 The EDTA-effect was reversible during its initial stage Perfus ons with plasma in wh ch Ca a d Mg had been remo ed by dialysis at pH 3 5 we e performed When an EDTA-effect had been induced w th th s type of perfusates t could be re ersed by addition of equ molar amounts of eithe Ca or Mg^{++} This indicates Ca^{++} and Mg^{++} to be equally important in order to assure normal permeability properties of the capillary membrane

The importance of calcium for the maintenance of normal capillary permeability was first demonstrated by Chambers and Zweifach (1940) on frog mesenterium Wilbrandt *et al* (1956) found that addition of EDTA induced a marked reduction in the effective osmotic pressure An increase of local capillary permeability was observed by Spector (1957) when sodium citrate was injected into the skin of rats Nicolaysen (1971 a, b) found that the pre vention of edema development in rabbit lungs was determined by either the [Mg] or the [Ca^{+}]+[Mg^{++}] Increased hydraulic conductivity was observed at low [Ca +], this effect could be reversed by Ca^{++} but not by Mg^{++}

A reduction of [Ca^{++}] (and [Mg]) causes a marked increase in the ion permeability in various types of tissue membranes (Curran 1961 Lipson *et al* 1965 Tidball 1964 Goldner 1967 Cuthbert and Maetz 1972) and of cells (Lyman 1945 Maizels 1956 Bolingbroke and Maizels 1959 Morill *et al* 1965 Morill and Robbins 1967) Addition of Ca^{++} usually

leads to recovery but there seems to be some disagreement about the role of Mg as a replacement ion for Ca^{++}

The site of action of low [Ca^{++}] on cellular membranes is not precisely known Most investigators call attention to the junctions between the epithelial or endothelial cells Withdrawal of Ca^{++} is said to weaken the cell adhesions (Chambers and Zweifach 1947 Hays et al 1965 Loewenstein 1971) Goldner (1967) however believes that the increased permeability caused by the exposure to EDTA is due to changes of the membranes of the epithelial cells rather than to changes at the cell junctions

No informations about the effect of changes in pH on the capillary permeability seem to exist The permeability of cells however has been shown to be influenced by variations of the hydrogen ion concentration in the extracellular fluid A loss of K from the sartorius muscle of frog to the incubation fluid caused by an increase in pCO_2 has been described by Fenn and Cobb (1934 and 1935) In the gall bladder epithelium a decrease or an increase in pH induce changes in the selective permeability to monovalent ions (Wright and Diamond 1968)

In the present investigation the effects of added EDTA and of pH variations on the capillary permeability have been studied To this end horse plasma perfusions of the swimbladder *rete mirabile* of the eel have been performed In this preparation a steady state transcapillary exchange could be established and the capillary permeability of various test substances continuously followed (Stray Pedersen and Steen 1975) The reversibility of the effects induced by EDTA or changes in pH was also investigated likewise the relative importance of Ca and Mg The results obtained in this investigation throw some light on the mechanisms of capillary permeability particularly of the function of Ca and Mg in this respect

Methods

The prepa ati n and perfu i n te hnique

For details the reader is referred to the papers of Stray Pedersen and Nicolaysen (1975) and of Stray Pedersen and Steen (1975)

Cannulation of the appropriate essels allowed simultaneous perfusion of the arterial n l ven us capillaries of the isolated re The preparation was perfused by horse plasma to which the ubstances under study were added Permeability values were obtained by comparing the concentrations c f these substances in the output w th those in the input perfusates

The *rete* was always perfused in a parallel fashion at constant flow (Unita Ierfusor Fum Braun Melsungen) The accuracy of the delivery of the pumps was better than 0 2 The perfusion pressures in both input cannulas were continu usly recorded by connecting a side branch of the cannulas to a Statham P 23 De transducer Optimal perfus on of the organ is obtained at an arterial input pressure of about 40 cm H_2O The flow was therefore adjusted to ach eve this pressure At such conditions the venous input pressure would be about 10 cm H O The output flow was determined by weighing the output accumulating over known periods of time Output samples of 1 ml were sufficient f r analys s

Pe fusates Horse plasma was obtained by centrifugation of heparinized blood (30 I U of Heparin (A L) per ml) Papaverine (0 1 mg ml) was added t) the perfusates in order to obtain a complete relaxation of the vascular smooth muscles (Stray Pedersen 1970) Papaverine in this dose does not affect the permeabil ty of the *rete* capillaries (Stray Pedersen and Steen 1975)

To study the effect of Ca and Mg EDTA (as Na EDTA) was added to the perfusates in varying concentrations At pH 7 4 the conditional constant of the Ca EDTA chelate is 10 and of the Mg EDTA chelate 10^{6} (Ringbom 1969) Hence an addition of equ mol r amounts of EDTA t plasma w ll lead to an almost com lete remo al of Ca and Mg

TABLE I Relevant physio-chemical data of the test-substances

Substance	MW	Molecular (ionic) rad us (Å)	$D_{s s}$ ($\times 10^5$ cm sec^{-1})	Olive oil water partition coefficients
(Water)	18	1.5	2 66 [1]	0 007 [6]
THO	20	1.5	2 44 [1]	do (?)
Na+	..3	0 95 (as nonhydrated)	1 48 as NaCl [ ]	Insoluble in oil
K+	39	1 33 (as nonhydrated)	1 917 as KCl [ ]	do
Ethanol	46	2.4	1..4 [3]	0 0.. [6]
Urea	60	2.64	1 38 [4]	0 00015 [6]
Antipyrine	188	4 1[b]	0 68	0 03 [6]
Sucrose	34	5 55	0.5 [4]	Insoluble in oil
Dextran-16 000	15 000–17 000	28	0 074 [5]	do
Dextran 75 000	60 000–90 000	60	0 036 [5]	do

Calculated as Stokes–Einstein rad us $r = RT/6\pi\eta DN$
[b] Calculated as equivalent sphere radius $r = (3M\ 4\pi\rho N)^{1\ 3}$
(M is the molecular weight ρ is the density N is Avogadro s number R is the gas constant, T is the absolute temperature and η is the viscosity of the sol ent)
Found by extrapolation from the diffusion coefficients of urea glucose and sucrose

References [1] Wang *et al* 1953 [ ] Handbook of Chemistry and Phys cs 1971 [3] Johnson and Babb 1956 [4] Longsworth 1954 [5] Personal communication from New England Nuclear [6] Collander and Bärlund 1933

Perfusates with low Ca/Mg-content were prepared by dialyzis for 40–60 h against deionized water at +4 C following acidification to pH 3 0–3 5 by addition of concentrated HCl To restore normal concentrations of Na^+ K^+ and Cl^- appropriate amounts of NaCl and KCl were added or the plasma was dialyzed against a Ca/Mg free Ringer solution By this procedure plasma with less than 0 005 mM/l of Ca and less than 0 00..5 mM l of Mg was obtained.

The effects of pH on the capillary permeability were stud ed by adding concentrated HCl and NaOH to the perfusates At extreme pH alues pl sma would flocculate and repeated filtration was necessary prior to the experiment.

Test m l ul The capillary permeab l ty of the follow ng substances were stud ed

Isotopes

^{24}Na (as NaCl Institutt for Atomenergi Kjeller Norway)
^{4}K (as KCl, do)
Antipyrine N methyl ^{1} C (Catalogue number NEC 398 New England Nuclear Boston, Mass.)
Urea-^{14}C (NEC 108 NEN)
Dextran carboxyl ^{1} C MW 15 000–17 000 (NEC 18A, NEN)
Dextran carbo yl ^{4}C MW 60 000–90 000 (NEC ..18B NEN)
Ethanol 1 ^{4}C (NEC-0 9 NEN)
THO (IFA Kjeller Norway)
Sucrose-^{4}C (NEC 100 NEN)

Non Isotopes

K as KCl

Some relevant phys al properties of the test molecules are listed n Table I

The substances to be in est gated were added to one of the perfusa.es. Care was taken to ma ntain the same osmolarity n the arterial and enous perfusat Hence addition of subst nces to one side was balanced by dd tion of N Cl or H O to the other K and THO wer used in all e periments to .. as reference molecules. Due to it easy determ ation, the cap llary exch ge of K^+ could followed througho t the experiments.

Radiochemical analysis. A 3 channel Auto Gamma Spectrometer and Tri Carb Liquid Scintillation Spectrometer (Packard Instrument Co.) were used for the measurements of radioactivity. Precautions were taken to count the isotopes separately. To achieve high accuracy the activity in the test perfusates was adjusted, when possible, to give at least 10 000 c.p.m. in the output samples.

Measurements of Na, K, Ca and Mg

The concentrations of Na, K and Ca were measured with an Eppendorf flame photometer. Within the range of normal plasma concentrations the determinations were accurate to within $\pm$ for Na and K and ± 3 for Ca. The measurements of Mg and in some instances also Ca were performed with an Unicam atomic absorption photometer. In routine performances of this method the concentrations could only be measured when [Ca] > 0.05 and [Mg] 0.0.5 mM/l. When necessary, samples were concentrated by evaporation whereby concentrations down to 0.005 and 0.00 5 mM/l of Ca and Mg respectively could be determined. By these methods the *total* concentration of the substances (ionized plus nonionized) were measured.

Determinations of ionic content of the rete tissue

Pieces of *rete* were removed and carefully squeezed on filter paper to absorb as much intravascular fluid as possible. After weighing, a known quantity of sulphuric acid was added and the solutions were lightly heated until total disintegration of the tissue. The chemical measurements were performed in this solution after addition of 00 µl H_2O. The other *rete* of the swimbladder (not perfused during the experiment) served as controls. (For details see Stray Pedersen and Nicolaysen 1975.)

Calculations of capillary permeability values

The basic assumptions and the evaluation of the permeability equations as well as the methods for the necessary corrections for shunts and leakages have been described earlier (Stray Pedersen and Steen 1975). Since these experiments were performed with parallel perfusion at conditions of equal flow, the permeability equation used was

$$P = \frac{W}{A_C \cdot L} \ln \frac{\Delta C_e}{\Delta C_L} \tag{1}$$

in which P = capillary permeability (cm sec^{-1}), W = flow rate (cm^3 sec^{-1}), A_C = capillary circumference (cm), L = capillary length (cm), ΔC = arterio-venous concentration difference of the test molecule at the input side, ΔC_L = arterio-venous concentration difference at the output side.

Since the size of the capillary surface ($S = A_C L$) was not measured in these experiments, we used the P·S product as a measurement of the capillary exchange

$$P \cdot S = P \cdot A_C \cdot L = W \ln \frac{\Delta C}{\Delta C_L} \tag{2}$$

The PS products of the different test molecules were calculated according to this equation and related to those of THO or K as permeability ratios (P/P_{THO} or P/P_K).

Results

The effect of Ca and Mg

The effect of different concentrations of EDTA in the perfusates was first studied. Perfusions with EDTA in concentrations of 10, 100, 1 0, 150, .00 and 300 of the concentration of Ca and Mg in the plasma perfusates were performed. No alterations in the capillary exchange were observed until the concentration of EDTA exceeded the equimolar concentration of Ca + Mg. Even with 100% EDTA the permeability of all the test substances remained constant during more than 2½ hours of perfusion. However, with 120 EDTA or more, a distinct effect on the permeability properties of the capillaries could be demon

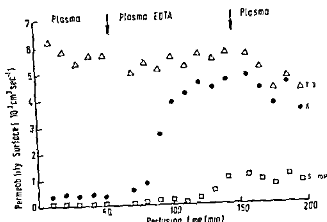


Fig. 1 The effect of EDTA. During the exposure to 6 mM EDTA both PS_{K^+} and PS_{S_0} increased whereas PS_{THO} remained fairly constant. Note the delay in the onset of the increase in PS_S compared to that of PS_{K^+}. At the end of the perfusion the mutual relationship between the capillary diffusion capacities was approximately identical to that between the corresponding free diffusion coefficients

strated. The latency for the effect to occur varied from experiment to experiment and seemed to be related to the rate of flow and the size of the *rete*. With 120 % EDTA the effect would usually appear within 30 to 90 minutes. When higher concentrations of EDTA were used the permeability changes would develop more quickly. With 200–300 % the EDTA-effect was usually apparant within 10–15 min.

Fig. 1–3 illustrate the capillary exchange of THO, K and sucrose or dextran 75 000 during perfusions with plasma containing 120 % EDTA. The addition of EDTA to the perfusates lead to a tremendous increase of the permeabilities of K^+ and the other water soluble molecules whereas the permeability of THO continued unchanged. In some experiments the P_{THO}/P_K ratio changed from 27 to about 1.3 during EDTA perfusion. This latter value is close to the ratio between the corresponding free diffusion coefficients in water. From the values obtained in 14 expts. with distinct (but not always maximal) EDTA effects the mean P_{THO}/P_K ratio was 2.9 when the EDTA-effects were most pronounced (Table II). As for the other watersoluble substances they seemed to follow K very closely. At maximal EDTA effects the mutual relationship between the exchange of water soluble substances was very similar to that between their diffusion coefficients in water (Table II).

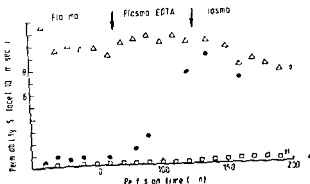


Fig. 2 The effect of EDTA. The onset of the increase in PS_{Dex} was significantly delayed compared to that of PS_{K^+}

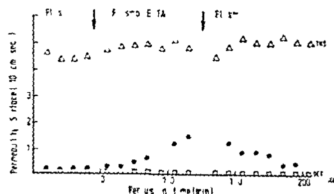


Fig. 3 The effect of EDTA During the perfusion with EDTA (120 of total plasma content of Ca + Mg) PS_{K^+} but *not* PS_{Dex} was increased Reperfusion with normal plasma lead to an almost complete reversibility

Sucrose was employed as test-substance in 5 expts In 3 of these the EDTA-effect developed slowly and the increase in sucrose permeability was delayed as compared to the development in K permeability (Fig 1) In this phase therefore a molecular size dependant restriction to transcapillary diffusion became temporarily apparent In the remaining two experiments the EDTA-effect developed more quickly and the permeability curves of K^+ and sucrose were parallel

The onset of the increase in the capillary exchange to macromolecules (dextran 16 000 and dextran 75 000) was always delayed as compared to the other water-soluble test molecules Usually the rise in the permeability of these substances could not be recognized until prior to the attainment of maximum permeabilities of smaller water soluble molecules The *rete* often showed signs of edema as judged from its macroscopical appearance within ½ h after the onset of the rise in dextran permeability At such circumstances the measurements of the permeabilities becomes inaccurate As for the lipid-soluble molecules they followed THO very closely The $P_{Ethanol}/P_{THO}$ and $P_{Antipyrine}/P_{THO}$ ratios remained constant throughout the EDTA perfusions even during the stages in which the *rete* was showing signs of edema and deterioration

Some of the EDTA treated *retia* were examined by light microscopy and by electron microscopy No changes in the capillary structure could be observed even in those *retia* showing a significant rise in dextran permeability (Fig 4)

The reversibility of the EDTA-effect

In many experiments approximately half of those presented in Table II attempts to reverse the permeability changes induced by EDTA were performed in the following manner When the EDTA-effect was definitely established as judged from the measurements of K during the experiment normal plasma perfusates were again employed Since both the time of latency for the occurrence of the initial permeability changes induced by EDTA and also the speed of the permeability changes after this point were highly unpredictable it was difficult to standardize these experiments

When the EDTA-effect developed very slowly however as was the case in three experiments (120 ₒ EDTA) it could be reversed One of these successful experiments is shown in

Table II Mean cap llary permeability ratios obtained during maximal effects of EDTA low and high pH

Perfusate	Number of experiments	Initial ratio P_{THO}/P_{K^+}	Mean ratios obtained when the effects were maximal							
			$\frac{P_{THO}}{P_{K^+}}$	$\frac{P_{Etha\ l}}{P_{THO}}$	$\frac{P_{A\ tipyrin}}{P_{THO}}$	$\frac{P_{24_{Na^+}}}{P_{K^+}}$	$\frac{P_{U\ a}}{P_{K^+}}$	$\frac{P_{S\ rose}}{P_{K^+}}$	$\frac{P_{De\ tran-16}}{P_{K^+}}$	$\frac{P_{D\ tra\ 75}}{P_{K^+}}$
Plasma + 4–12 mM/l EDTA	14	?7	[illegible]9	1 03	0 90	0 82	0 83	0 34	0 09	0 05
Plasma pH 3 5 3 7	4	21	1 2	0 97				0 41	0 12	0 09
Plasma pH 9 2 10 0	5	32	1 6		0 81				0 11	0 06
Corresponding ratios between the free diffusion coefficients in water at ?0 C			1 27	0 5	0 8	0 74	0 72	0 ?7	0 038	0 018
Normal permeability ratios of the retial capillaries (Stray Pedersen and Steen 1975)			36 2	1 02	0 86	0 78	0 94	0 31	—	—

effect had been partially reversed the $[K^+]/[Na^+]$ ratio was seen to lie between the plasma value and the normal value of *rete* tissue

Experiments with dialyzed plasma

From the results obtained in the experiments mentioned above it must be concluded that the presence of Ca^{++} *or* Mg^{++} *or* both is needed to maintain the permeability properties of the rete capillaries A study of the relative importance of these ions would necessitate perfusions with plasma from which Ca^{++} or Mg^{++} had been removed Such perfusates could be obtained by dialysis of plasma following acidification The influence of pH on the binding of calcium and magnesium was investigated in a sequence of dialyzis experiments Varying amounts of HCl NaOH or EDTA were added to plasma and Ringer solutions which were dialyzed against tap water or deionized water pH and the concentrations of Na K Ca and Mg were measured before and after the dialysis During the dialysis of plasma flocculation of proteins (globulins) would occur The plasma dialyzate was therefore centrifuged and the analyses were performed in the supernatant In some instances the content of calcium in the precipitate was also determined Prior to this the precipitate had been repeatedly washed in deionized water and centrifuged and then dissolved in a Ca and Mg free saline solution Some typical results from these experiments are shown in Table IV

As expected more than half of the total Ca and Mg was removed when plasma of normal pH was dialyzed against deionized water (Dialysis 1 in the table) Most of the Ca and Mg was found in the supernatant (albumin fraction) almost nothing could be detected in the precipitate (globulin fraction) (Dialysis 2)

To our great surprise an *increase* of the content of both Ca^{++} and Mg^{++} was observed when plasma of normal pH was dialyzed against tap water (Dialysis 6) In spite of a 20 fold higher concentration of Ca and Mg in plasma than in water both substances were absorbed by the plasma *i e* against the apparent gradient In the Ringer solutions Ca and Mg existed as free ions only and were hence effectively removed during dialysis against deionized water as well as against tap water (Dialysis 8)

As for the EDTA-containing solutions it should be emphasized that this chelating agent was always added in excess to the content of total calcium and magnesium However when added to plasma which was then dialyzed against tap water (Dialysis 7) only about 30% of the calcium and magnesium was lost *i e* the content of Ca and Mg in the dialyzate was significantly greater than the fraction representing protein bound Ca and Mg (see Dialysis 1) This finding could not be explained by an interference between the EDTA added and Ca and Mg ions outside the dialyzing bag since similar results were obtained when dialysis was carried out against deionized water (Dialysis 3) As seen from the results obtained with Ringer solutions (Dialyses 8 and 9) a significant amount of Ca and Mg was retained when EDTA had been added (Dialysis 8) It was therefore reasonable to suggest this to be due to permeability properties of the dialyzing bag the membrane of which must offer considerable restriction to the diffusion of the EDTA-chelate complexes as compared to that of the free ions of Ca and Mg Unfortunately no information about the pore sizes of the dialyzing membrane employed could be obtained from the producer At all events by comparing the results obtained in Dialyses 6 7 and 9 it must be concluded that EDTA

TABLE IV The content of Na K Ca and Mg in different plasma and Ringer solutions before and after dialysis — = not measured

Performance of the dialysis	Dialysis number	Solutions	pH		Na mE/l		K mE/l		Ca mE/l		Mg[a] mE/l	
			Bef	Aft	Bef	Aft	Bef	Aft	Bef	Aft	Bef	Aft
Dialysis against deionized water (6 hrs +4 C)	1	Plasma normal pH (supernatant)	8 2	7 6	128	0 4	3 4	0 2	5 2	2 1[a]	1 5	0 5
	2	Plasma normal pH (precipitate dissolved in saline)	do	5 0	do	—	do	—	do	0 2[a]	do	0 1
	3	Plasma + 4 mM/l EDTA (supernatant)	7 8	7 5	—	—	—	—	5 1	3 4	—	—
	4	Plasma low pH (do)	3 7	3 9	—	0 2	3 2	0 1	5 1	0[b]	1 4	0[b]
	5	Plasma high pH (do)	9 7	9 5	—	0 2	3 2	0 1	5 2	5 0	1 2	1 1
Dialysis against tap water (38 hrs +4 C)	6	Plasma normal pH (supernatant)	7 6	7 4	121	0 4	3 4	0 1	4 8	5 0	1 4	1 6
	7	Plasma + 4 mM/l EDTA (do)	6 9	7 2	127	0 3	3 2	0 1	4 8	3 4	1 3	1 0
	8	Ringer	4 1	5 9	140	0 2	4 5	0 1	4 2	0 1	4 5	0 1
	9	Ringer + 4 mM/l EDTA	3 6	6 8	141	0 3	4 4	0 2	4 2	1 7	2 3	0 3
	10	Plasma low pH (supernatant)	3 9	4 0	—	0 1	3 3	0 1	4 9	0 1	1 3	0 1
	11	Plasma high pH (do)	9 6	8 7	—	0 4	3	0 1	4 8	7 3	1 2	2 0
		Tap water	6 2		0 2		0 1		0 2		0 1	
		Deionized water	5 8		—		—		0[b]		0[b]	

[a] Measured directly with Atomic absorption photometer
[b] Measured with Atomic absorption photometer after evaporation of the sample (see Methods)

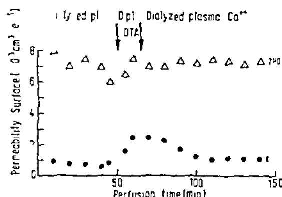


Fig 8 The reversibility of the EDTA effect with Ca^{++} The EDTA-effect developed *rapidly when 0 8 mM/l EDTA was added* to the dialyzed plasma A reversibility was obtained during the final perfusion with dialyzed plasma containing 0 8 mM/l EDTA and 2 5 mM/l of $CaCl_2$

by no means combines quantitatively with the protein bound Ca and Mg in plasma At this pH therefore the binding of Ca and Mg to the plasma proteins must be stronger than those between Ca/Mg and EDTA This is rather surprising considering the very low conditional constants of the Ca and Mg EDTA complexes

When the dialysis was performed at pH values below 4 0 Ca and Mg were effectively removed (Dialyses 4 and 10) When dialyzed against deionized water (Dialysis 4) the concentrations of these substances became unmeasurable *i e* [Ca] < 0 005 and [Mg] < 0 0025 mM/l On the other hand a great increase in the Ca/Mg binding capacity of the proteins was observed when the plasma had been alkalinized During dialysis against tap water at pH of about 9 5 Ca^{+} and Mg^{+} were absorbed from the water giving a 50 % increase in the content of both substances (Dialysis 11) When deionized water was used less than 10 % of the total Ca + Mg was lost (Dialysis 5) whereas more than 50% was lost at normal pH values (Dialysis 1)

Perfusions with dialyzed plasma Reversibility of the EDTA-effect with Ca^{++} or Mg

Experiments were performed with plasma which had been dialyzed at pH 3 0–3 5 and to which appropriate amounts of NaCl and KCl had been added (but *not* Ca or Mg) Such perfusions could last for 4–5 h No changes in the permeability properties of the capillaries could be observed

Several attempts were made to reverse the EDTA induced effects by addition of either Ca or Mg This was performed as follows The *rete* was initially perfused with dialyzed (Ca/Mg free) plasma Then an EDTA-effect was induced by addition of 0 8 mM/l EDTA The EDTA-effect would usually appear within 10 to 15 minutes Now the perfusate was exchanged with one of the two following solutions

1 Dialyzed plasma + 0 8 mM/l EDTA 2.5 mM/l Ca +
2. Dialyzed plasma + 0 8 mM/l EDTA 2.5 mM/l Mg

In 4 expts only we succeeded in changing the perfusate during the initial phase of the EDTA-effect In one experiment with each perfusate the *EDTA-effect was actually reversed* (Fig. 8 and 9) In the two other experiments the introduction of the Ca/Mg-containing per

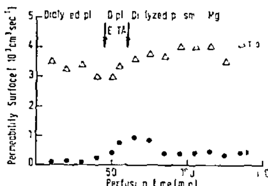


Fig 9 The reversibility of the EDTA-effect with Mg^{++} The EDTA-effect was elicited by addition of 0 8 mM/l of EDTA to the dialyzed plasma. A reversibility was obtained with the same type of perfusate to which 2 5 mM/l of $MgCl_2$ had been added

fusates happened to occur somewhat later in the development of the EDTA-effect *i e* just prior to the increase of the dextran permeability In these experiments the increase of the permeabilities continued without any delay as was also the case in the other non successful experiments of this type

Discussion

The normal permeability relationships of the capillaries in the *rete mirabile* have been presented in an earlier paper (Stray Pedersen and Steen 1975) The capillary membrane was found to be 36 times more permeable to THO than to K^+ (Table II) The mutual relationship between the permeabilities of the smaller water soluble molecules was almost identical to those between the corresponding free diffusion coefficients The permeability of the lipid soluble molecules were proportional to their lipid solubility These findings indicated that the capillary membrane has three pathways for transcapillary diffusion One with lipoid character one allowing water only and one that allows smaller hydrophilic molecules and ions (including water) to pass without progressive size or charge dependent restriction The intercellular junctions or slits are candidates for this latter pathway The two first mentioned pathways are most likely situated in the endothelial cell membrane

The addition of EDTA produced characteristic and reproducible effects on the capillary permeability in the *rete* Similar responses to EDTA were also obtained in our investigation on the gill membrane (Steen and Stray Pedersen 1975) Possible effects of the decreased pH following the addition of EDTA to the perfusates were excluded by low pH perfusion experiments mimicking the pH effect caused by EDTA There is a possibility that the permeability changes were caused by chelation of EDTA to other unidentified molecules but we consider this unlikely We feel therefore confident that the main effect of adding EDTA is to reduce the concentration of calcium and magnesium ions in the perfusate and in the tissue

The EDTA-effect consisted of 3 consecutive phases

1 An initial phase in which the capillary permeability to smaller hydrophilic molecules increased dramatically whereas the permeability to THO and lipid-soluble molecules was not affected

2. A second stage in which the capillaries became significantly permeable to macromole

cules The fact that the $P_{Dextran}/P_{K^+}$ ratios were higher than the ratios between the corresponding (theoretical) diffusion coefficients in water must be ascribed to inhomogenities of the dextran fractions employed The onset of increased dextran permeability seemed to represent the point where the EDTA-effect no longer could be reversed

3 A final phase in which the *rete* showed signs of edema and deterioration

The time needed for these phases to develop depended on the EDTA-concentration and the perfusion rate

The increase in capillary permeability caused by removal of Ca^{++} and/or Mg^{++} is generally assumed to be due to a weakening of the cellular adhesions *i e* making the intercellular slits or junctions to behave as wider channels Three lines of evidence supports the view that the endothelial cell membrane represents the primary site of action of Ca and Mg

1 If the effect of the removal of Ca and Mg should be a widening of the intercellular slits *or* an increase in the total slit area to such extent as to give 20-fold increase of K permeability then a significant rise in the exchange of THO should be expected This was not found

2 A normal *rete* contains almost equimolar amounts of K^+ and Na (Stray Pedersen and Nicolaysen 1975) whereas *retia* treated with EDTA showed an ionic composition very similar to that of plasma (Table III) In the experiments in which the EDTA effect was reversed, the [K] [Na]- ratios were more similar to the values of normal retia (Table III)

3 In some experiments (Fig. 1) a transient restriction of sucrose diffusion as compared to K diffusion was observed during the initial phase of the EDTA-effect This could indicate a recruitment of new pores which were initially too small to allow the passage of sucrose

As for the second phase of the EDTA-effect—characterized by an increased capillary permeability to dextrans—this is probably due to changes at the levels of the junctions. However as seen from Fig 4 the ultrastructure of dextran permeable capillaries appeared to be normal without widenings of the junctional gaps The same was also observed in capillaries of the rabbit lung sufficiently exposed to EDTA to give edema (Hovig *et al* 1971) This does by no means exclude that great changes may have taken plase *in vivo* Earlier studies indicate that the junctional gaps in normal retial capillaries might be considerably wider (average diameter ~ 1 300 Å) than seen on the micrographs (Stray Pedersen and Steen 1975) The fact that these channels offer substantial restriction to the diffusion of high molecular dextrans is most probably due to the presence of a fibrillar matrix or cement substance which functions as a filter When Ca (and Mg) is removed this matrix may change in such a way as to create pathways through which the dextrans may pass The fact that it was possible to reverse the EDTA-effect by addition of equimolar amounts of either Ca or Mg to the Ca Mg free" plasma containing 0 8 mM/l EDTA (Figs 8 and 9) indicated these ions to be of qualitatively and perhaps also of quantitatively equal significance for the maintenance of normal permeability characteristics

The EDTA-experiments showed that very small amounts of Ca and Mg are necessary for maintenance of normal permeability characteristics This is in agreement with results in earlier investigations performed on frog mesenterium (Chambers and Zweifach 1940) frog gastric mucosa (Sedar and Forte 1964) and rabbit lungs (Nicolaysen 1971 a b) In our experiments the lowest EDTA-concentration which elicited permeability effects was 120

of the total plasma content of Ca ++Mg++ This corresponds to [Ca^{++}] and [Mg^{+}] in plasma of about 7×10^{-3} and 1×10^{-3} mM/l respectively In the dialyzed plasma which caused no permeability effect [Ca^{++}] was less than 5×10^{-3} and [Mg^{++}] less than 2.5×10^{-3} mM/l The "critical plasma concentrations of Ca^{++} and Mg^{++} therefore appears to lie between these values This is in fair agreement with values found by Nicolaysen (1971 a b) for the rabbit lung However it must be emphasized that these calculations of the concentrations of Ca + and Mg^{++} in the presence of EDTA in plasma are based on the assumption that EDTA would combine effectively and equimolarily to all of the Ca and Mg in plasma According to the results obtained in our dialysis experiments this assumption may be rather doubtful

In contrast to the EDTA-effect the changes in the capillary permeabilities caused by extreme hydrogen ion concentrations in the perfusates occurred very abruptly When the pH was brought close to the critical values only moderate shifts in pH were necessary to elicit the effects thus indicating an all-or none-effect For instance at pH 3 9 and 8 8 normal permeability properties were maintained while at pH 3 5 and 9 4 the effects were dramatic (Fig 5 and 7) The effects caused by high or low pH could not be reversed The main pattern of the permeability changes (Table III) was similar to that obtained during EDTA-exposure This could indicate that the mechanisms of the permeability effects of EDTA and high/low pH are the same The fact that addition of calcium induced a delay in the response to low pH (Fig 6) is supporting evidence to this proposal

We suggest that our findings can be explained in the following way

Under normal conditions Ca^{++} is associated with the cell membrane and exerts its function by its charge the volume it occupies and possibly by allosteric effects It may therefore function as a positively charged plug When the *rete* is exposed to excess EDTA the Ca + ions will leave the membrane and become bound to EDTA At the same time EDTA will enter the endothelial cells and Ca^{+} will leave it As more and more Ca plugs are removed the cell membrane looses progressively both its selectivity and its restriction to diffusion At a certain stage all Ca + will be gone and the cell membrane can be compared to a lipid protein sheeth with a certain porosity Since Ca^{+} also has a structure-stabilizing function the cell to cell adhesion will be destroyed during such conditions During an early stage when some Ca is still present, the process is reversible

We know that at a low pH the affinity of plasma proteins to Ca is lowered at a high pH it is increased In artificial (phospholipid) membranes Seimiya and Ohki (1973) have shown the degree of Ca absorption to be highly influenced by pH It seems reasonable that the proteins (and phospholipids) of the endothelial cell membrane would behave similarly A low pH will therefore cause release of Ca^{+} from the cell membranes whereas a high pH may strengthen the Ca + protein bonds so that Ca can no longer act as a charged plug Alternatively high pH may increase the affinity of plasma proteins so that they in fact act as EDTA The reason why pH-effects give a more rapid effect than EDTA may be that H ions pass the endothelial cell membrane much more rapidly than do Ca + or EDTA In frog skeletal muscle H has been found to diffuse more than 100 times faster than K^{+} (Woodbury *et al* 1968)

Our observations in the dialysis experiments concerning the effects of high and

are in agreement with data reported by Pedersen (1969). He found a linear relationship between the fraction of ultrafiltrable Ca and pH in plasma within a pH range of 6 and 9. Extrapolation from this curve indicates that 100% of the calcium content would be ultrafiltrable at pH 4 and 0% at pH 10. This conforms to the results obtained in Dialyses 4 and 5 in Table IV.

Plasma $[Ca^{++}]$, $[Mg^{++}]$ or $[H^{+}]$ necessary for permeability changes to develop in the present investigation, have never been described in systemic blood *in vivo*. Extreme $[H^{+}]$, however, has been found locally in many organs, such as for instance exercising muscles (Barcroft 1968). It has also been postulated that the capillary permeability of skeletal muscles increase during exercise (Bolme and Edwall 1971). The fact that the significant shift in venous pH of working muscles is accompanied by marked changes in $[Na^{+}]$ and $[K^{+}]$ (Barcroft 1968) is interesting in this connection. On the basis of the present investigation it is tempting to suggest the increase in $[K^{+}]$ observed is due to locally decreased pH and thereby removal of Ca^{++} and Mg^{++} from the tissue, including the capillary membranes. In this way local changes in pH in skeletal muscles and perhaps also in other organs may function to regulate the capillary permeability.

The author has been a Research Fellow of the Norwegian Research Council for Science and the Humanities. Financial support from this Council and from the Nansen Foundation is gratefully acknowledged. I also wish to thank Prof. J. B. Steen for all advice and help during the performance of this work, and Dr. G. Nicolaysen and Prof. B. A. Waaler for their valuable comments on the manuscript. Thanks are also due to Mrs. S. Schram and Mrs. G. Åkervik for skilful technical assistance.

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are in agreement with data reported by Pedersen (1969). He found a linear relationship between the fraction of ultrafiltrable Ca and pH in plasma within a pH range of 6 and 9. Extrapolation from this curve indicates that 100% of the calcium content would be ultrafiltrable at pH 4 and 0% at pH 10. This conforms to the results obtained in Dialyses 4 and 5 in Table IV.

Plasma $[Ca^{++}]$, $[Mg^{++}]$ or $[H^+]$ necessary for permeability changes to develop in the present investigation have never been described in systemic blood *in vivo*. Extreme $[H^+]$ however has been found locally in many organs, such as for instance exercising muscles (Barcroft 1969). It has also been postulated that the capillary permeability of skeletal muscles increase during exercise (Bolme and Edwall 1971). The fact that the significant shift in venous pH of working muscles is accompanied by marked changes in $[Na^+]$ and $[K^+]$ (Barcroft 1968) is interesting in this connection. On the basis of the present investigation it is tempting to suggest the increase in $[K^+]$ observed is due to locally decreased pH and thereby removal of Ca^{++} and Mg^{++} from the tissue including the capillary membranes. In this way local changes in pH in skeletal muscles and perhaps also in other organs may function to regulate the capillary permeability.

The author has been a Research Fellow of the Norwegian Research Council for Science and the Humanities. Financial support from this Council and from the Nansen Foundation is gratefully acknowledged. I also wish to thank Prof. J. B. Steen for all advice and help during the performance of this work, and Dr. G. Nicolaysen and Prof. B. A. Waaler for their valuable comments on the manuscript. Thanks are also due to Mrs. S. Schram and Mrs. G. Åkervik for skilful technical assistance.

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in human infants (Aperia *et al* 1972, Aperia *et al* in press, McCance and Widdowson 1957) piglets (McCance and Widdowson 1957) rats (Trimble 1970) and dogs (Kleinman and Reuter 1974)

In the present study the natriuretic response to a saline infusion has been determined in lambs of two age groups The response has been related to developmental changes in intra renal hemodynamics The relation of salt excretion to the hemodynamics of the kidney was mainly motivated by the importance of blood flow for the control of intrarenal physical forces under normal physiological conditions partly also by the possible effect of intra renal blood flow distribution on total salt excretion.

Material and Methods

Prepa ation of animal Studies were performed in 13 lambs of both sexes nine aged 5–_8 days and four aged 48-57 days All lambs were given possibility of sucking or free fluid intake up to about 2 h before the experiment, each of which was performed on approximately the same time of the day The animals were anesthetized with penthotalsodium (0–25 mg/kg b wt.) intubated for ventilation (oxygen and nitrous oxide) with an Engstrom respirator and were given atropine (0 0 mg.kg b wt.) and pancuroniumbromide (Pavulon 0 02–0 03 mg/kg b wt) for muscle relaxation They were placed on a heat controlled table to maintain rectal temperature at 37.5–38 0 C.

Cannulas were inserted into one brachial and one femoral vein for infusions Both femoral arteries were catheterized with an end hole polyethylene catheter One was introduced into the left ventricle for blood pressure recordings and for injections of microspheres and Cardio-green The position of the catheter tip in the left ventricle was verified by the shape of the pressure curve Blood samples were drawn from the other femoral artery Both ureters were dissected after a suprapubic incision and can nulated (infant feeding tube no 5) for the purpose of urine collection

Exp rimental p otocol For the determinations of glomerular filtration rate (GFR) and urinary sodium excret on ($U_{Na}V$) standard clearance techniques were used The stud es were performed during the cont nuous infus on of Inulin (10% Laevosan Ges Linz, Austria) (0 15 ml min/kg b wt) The infusion was started 60 min before the f rst urine collect on period and preceded by a prime dose of 0 5 ml/kg b wt Urine was collected during 15 min periods and arterial blood samples were taken at the mid point of each collection period Following two control periods an ntravenous infusion of a sal ne solution (Na 147 mEq l) was added During the first 45 m n experimental period it was administered at a rate of 10 ml/h kg b wt The infusion rate during the following two periods was 0 and 30 ml/h/kg b wt. respecti ely At the end of each experiment (45 min) a total saline volume of 0 75 5% and 4 5 of b wt. espect ve ly had then been injected Calculations from urine collected during the last 15 min of each period was registered Blood flow determinat ons were performed mmediately before and after the progressi iso-tonic ECV expansion

H mody am c stud es Distribution of renal cortical blood flow befor and afte olume expans on was determined w th rad oact ve microspheres (3 M Company St Paul, Minnesota USA) labelled with d fferent isotopes (Cr^5 and $Ce^{1\,4}$ respecti ely) The microsphe es measuring 15 / m in diameter were suspended in 10 de tran and a few droplets of Tween 80 as a det g nt A sample of 1 ml (ncluding about 400 000 m rospheres) was tr ated with ultrason c agitation to a o d adh ren and then injected into the left ventricle during 5–10 s. In o der to d s rmine absolute t ssue flow the card ac outp t was recorded w th a dy d lut on techniq e (Cardiogreen) prev ously desc bed (Aper *et al* 1974) Blood pressure was recorded on a Grass polygraph a Stath m pressure transduce

An l s s of tissu Just before sacrifici g th animals Ind a nk (up to 0 ml) was injected to the aorta just abo e the entrance of the renal arteries The ink was gi en for marking the glomeruli to facil tate cortical sl cing The kid eys were then remo ed, weighed a d cut lo g tud nally Three to four about 1 mm thick sag tt l renal t sue blocks w re sect oned The cortex w s cut out of e ch block w th a th n and sharp kn f under a d ssect on m croscope into three circumf rential p eces—an outer a middle and an i ner cort cal l yer each occupy ng about 10 65 and _5 respect ely of the total cortical olume The inner cort l sl ces corresponded app oximately to the zone where the m jo ity of the ju tamedullary neph ons are s tuated Each cortical slice was w ghed wet a d co nted in a P cka d A to-gamma spectr meter

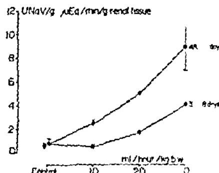


Fig 1 Mean urinary sodium excretion per gram renal tissue (µEq min g) (±S E.) during isotonic saline in fusion with three succeedingly in reasing infusion rates The recordings were made when each infusion rate had been maintained for 45 min

Analytical methods The chemical analyses of inulin in serum and urine were performed according to the Anthron method described by Hilger *et al* (1958) Serum total protein concentration was analyzed with a rephractrometric method Sodium was determined with a flame photometer (Eppendorf)

Calculations The regional cortical tissue flow was counted from the formula q CO (Q wt) where q and Q is the count rate in the individual slices and that injected into the animals respectively CO is the cardiac output and wt is the weight of the individual slices

Results

Urinary sodium excretion The saline infusion resulted in a continuous increase of urinary sodium excretion in all animals (Fig 1) The increase was out of proportion to the change of GFR (Fig 1 2 a b) There was however a difference in the degree of response in the two groups studied In the younger lambs the natriuresis was much less marked at each level of ECV-expansion

General effects of ECV-expansion There was no apparent effect of ECV-expansion on cardiac output or left ventricular systolic blood pressure in any of the animals studied (Fig

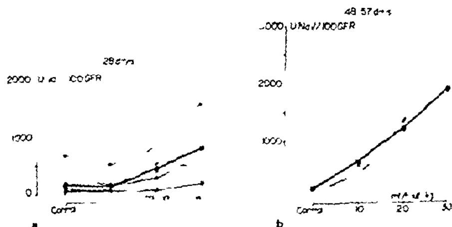


Fig. a b Urinary sod um excret on (µEq m n) per unit f ltered load during isotonic sal ne infusion with three succeedingly increasing infus on rates The small f lled circles connected with thin l nes represent the individual recordings in the two age groups (5 8 days (Fig a) and 48 57 days (F g b)) the large filled c rcles connected with heavy l nes represent the correspond ng mean values.

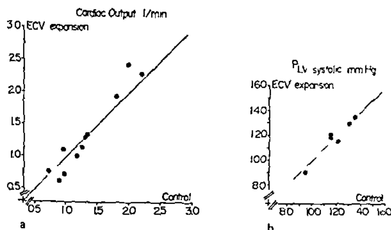


Fig 3a b Effects of saline infusion (4 5% of b wt) on cardiac output (Fig 3a) and left ventricular systolic blood pressure (Fig 3b) Line is one of identity

3a b) Nor was there any effect on the serum sodium value (Fig 4) The ECV-expansion resulted in a significant fall ($p < 0.05$) in serum protein concentration in both age groups (Fig. 5) The average fall in serum protein concentration was somewhat larger in the younger animals but the difference between the two age groups was not significant The fall in serum protein concentration was paralleled by a fall in hematocrit (Fig 6)

Renal hemodynamics The ECV-expansion had different effects on renal hemodynamics in the two age groups studied As shown in Fig 7a b the blood flow to the outer and mid cortex did not change much in the 5–28 day old lambs (Fig 7a) while the blood flow to the inner cortex increased somewhat (Fig. 7b) In the 48–57 day old lambs there was an increase in blood flow to all cortical layers (Fig 7a b) The relationship between inner and outer cortical blood flow increased somewhat more in the younger lambs than in the older lambs (Fig. 8)

Glomerular filtration rate The values for glomerular filtration rate (GFR) found during the control periods were in accordance with those previously recorded in this laboratory in

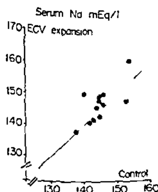


Fig. 4 Effect of saline infusion (4 5 of b wt) on serum sodium concentration Line is one of identity

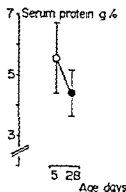


Fig 5

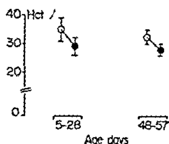


Fig 6

Fig 5 Effect of saline infusion (4 5 of b wt) on mean serum protein concentration in the two age groups. Unfilled circles represent control values ($\pm$S D) filled circles represent values when saline had been infused ($\pm$S D)

Fig 6 Effect of saline infusion (4 5 of b wt) on mean hematocrit in the two age groups Unfilled circles represent control values ($\pm$S D) filled circles represent values when saline had been infused ($\pm$S D)

developing lambs (Aperia *et al* 1974) Thus there was an exponential increase in glomerular filtration rate per gram renal tissue with increasing age Fig 9 demonstrates a comparison between the GFR before and after ECV-expansion in each animal studied The degree of volume expansion used had little effect on the GFR in either age group

It thus appears as if there is a discrepancy between the effect of ECV-expansion on renal b d flow and on glomerular filtration rate in at least the older animals This discrepancy is confirmed in Fig 10 where the relationship between GFR and cortical blood flow has been examined in both age groups studied In the younger animals there was no significant

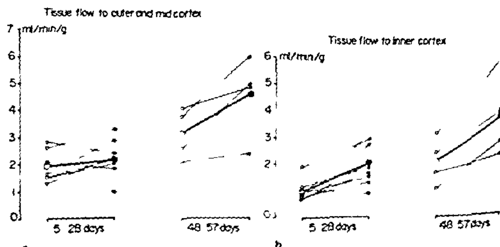


Fig 7a b Individual changes of blood flow per gram renal tissue (ml m n g) to outer and mid cortex (Fig 7a) and to inner cortex (Fig 7b) in the two age groups before and after saline infusion (4 5 of b wt). The small unfilled circles represent control values the small filled circles represent flow values when saline had been infused the corresponding large circles represent mean flow values respectively

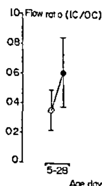


Fig 8

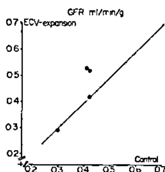


Fig 9

Fig 8 The inner to outer mean cortical blood flow ratio in the two age groups before and after saline infusion (4 5 of b wt) Unfilled circles represent control values (±S D) filled circles represent the flow ratio when saline had been infused (±S D)

Fig. 9 Effect of saline infusion (4 5 of b wt) on glomerular filtration rate per gram renal tissue (GFR/g) Line is one of identity

difference between the GFR to cortical blood flow quotient before and after expansion while in the older animals there was a significant decrease ($p < 0.05$) in the quotient during ECV-expansion Since the effect of ECV-expansion on hematocrit was similar in the two age groups it can be concluded that the GFR to plasma flow ratio *i e* the filtration fraction was more reduced in the older lambs

Discussion

The use of the microsphere method for intrarenal blood flow determinations has been evaluated previously (Katz *et al* 1971 Slotkoff *et al* 1971) The method has been found to be reliable under standardized conditions The microspheres are supposed to mainly indicate the distribution of red cells that are subject to axial streaming It might thus be influenced by changes in hematocrit Therefore the reliability of the method for repeated determinations during ECV-expansion has been discussed (Blantz *et al* 1971 Wallin *et al* 1971) The main purpose of this study however has been to relate the intrarenal tissue flow before and after volume expansion to age The possible error of the microsphere method should therefore influence the results to the same extent in the two age groups

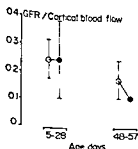


Fig 10 The quotient between glomerular filtration rate (GFR) and cortical blood flow in the two age groups before and after saline infusion (4 5 of b wt.) Unfilled circles represent control values (±S D) filled circles represent the ratio when saline had been infused (±S D)

A number of recent studies have demonstrated that the response to a salt and fluid load is suppressed in several newborn species (Aperia *et al* 1972 Aperia *et al* in press Kleinman and Reuter 1974 McCance and Widdowson 1957 Trimble 1970) In the present study it was found that the renal response to an isotonic ECV-expansion is age dependent also in lambs The urinary sodium excretion per unit filtered load was markedly lower in the 5–28 day old lambs than in the 48–57 day old lambs

Changes in intrarenal physical forces that will result in inhibition of proximal tubular reabsorption of sodium are thought to be an important mechanism for the natriuretic response to an ECV-expansion in adult animals (Brenner *et al* 1971 Grandchamp and Boulpaep 1974 Martino and Earley 1967 Windhager *et al* 1969) These forces are governed by the transtubular oncotic and hydrostatic pressure gradients The changes of the gradients are mainly determined by the oncotic and hydrostatic pressures in the peritubular capillaries The oncotic pressure is dependent on the serum protein concentration and on the filtration fraction The hydrostatic pressure in the peritubular capillaries is dependent on the arterial blood pressure and on the relationship between the resistance in the afferent and the efferent arterioles It is thus apparent that changes in intrarenal hemodynamics will have great consequences for the reabsorption of sodium and water by exerting an effect on intrarenal physical forces

In the present study the following parameters that could influence intrarenal physical forces have been followed the serum protein concentration the filtration fraction and the arterial blood pressure The serum protein concentration fell markedly in both age groups In the younger animals there was a slight and insignificant increase in both GFR and total cortical blood flow In the older animals there was also a slight increase in GFR while there was a fairly pronounced increase in the tissue flow to all cortical layers following the ECV-expansion This resulted in a significant fall in the GFR to cortical blood flow quotient During ECV-expansion the hematocrit decreased to about the same extent in all animals (Fig 6) The decrease in the filtration fraction must therefore have been more pronounced in the oldest age group The fall in serum protein concentration was equivalent in the two age groups It can therefore be concluded that the serum protein concentration and thus the oncotic pressure in the peritubular capillaries was more reduced in the older animals than in the younger animals following the expansion This difference might at least in part explain the difference in fractional salt excretion in the two age groups of animals No change was recorded in the arterial blood pressure during the ECV-expansion It can, however not be excluded that the expansion resulted in changes in intrarenal hydrostatic pressure The change in filtration fraction induced in the older animals suggests that the relationship between the vascular resistance in the afferent and in the efferent arterioles was also changed The fact that the renal blood flow increased while the cardiac output remained fairly constant during the expansion also indicates that the expansion induced some specific changes in the renal vascular system.

Although the importance of physical forces is well established it has been suggested that other factors will also contribute to the natriuretic response induced by an ECV-expansion. One of those is a functional redistribution of the filtrate (Barger 1966 Earley and Friedler 1965) This would mean that the proportion of the filtration occurring in the superficial

nephrons will increase in comparison to the filtration in the deep nephrons Since it is suggested that the short proximal tubules of the outer cortical nephrons will reabsorb less sodium than the long proximal tubules of the juxtamedullary nephrons such a redistribution to the superficial nephrons will result in a natriuresis (Horster and Thurau 1968 Jamison and Lacy 1971) In the present study the ECV-expansion produced a relative increase in blood flow to the inner cortex in the very young lambs The functional significance of an intrarenal blood flow redistribution is however unclear (Barratt *et al* 1973 Stein *et al* 1972) There are today no uniform data stating a constant relationship between GFR and glomerular blood flow in the juxtamedullary nephrons In fact it has been shown with the use of the ferrocyanide technique that in chronically salt loaded rats there is a functional redistribution of the filtrate to the outer cortex that is more pronounced in the younger animals (Baines 1973) The intrarenal blood flow distribution before and after ECV-expansion has previously been studied in 1–30 day old puppies (Kleinman and Reuter 1974) Since however puppies might be in an earlier stage of renal development than lambs of corresponding age (Aperia *et al* 1974) and also because of different cortical sectioning the results from that study (Kleinman and Reuter 1974) cannot be directly compared with the present study

The present results have not unequivocally pointed to the factor responsible for the suppressed natriuretic response to ECV-expansion in the very young animals It is quite likely that the inability to decrease the filtration fraction in the youngest animals contributed to the low salt excretion There are however several factors that are not directly dependent on renal hemodynamics that might also be responsible for the poor natriuretic response to an ECV-expansion Such factors as the renin angiotensin system the prostaglandins enhanced distal tubular sodium reabsorption have however not been evaluated in the present study which primarily aimed at clarifying the role of intrarenal hemodynamics for the control of salt excretion during development

Supported by grants from the Swedish Medical Research Council (B74 19X 3644-01B) the Prenatal Research Fund of Expressen and the Research Funds of the Karolinska Institute

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Acta physiol scand 1975 94 451–458

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On the Noradrenaline Loading in Axonal Amine Storage Granules in Rat Crushed Sciatic Nerves[1]

By

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Received 15 January 1975

Abstract

Dahlström, A., J Häggendal and P A Larsson *On the noradrenaline loading in axonal amine storage granules in rat crushed sciatic nerves* Acta physiol scand. 1975 94 451–458

The increase of NA in rat sciatic nerve above a crush was investigated. The transported amounts of NA did not increase quite linearly with time but more NA was found above the crush at 6 and 12 h than would be expected from the 3 h value One possible reason for this phenomenon—an increased NA load of the accumulated axonal granules—was investigated by 2 types of double crush experiments. One type involved simultaneous double crushes 1–1.5 cm apart. The increase in NA in the isolated segment 6 h after crush indicated that the axonal amine storage granules had increased their NA load by about [illegible]. In the second type ("delayed double crushes") a distal crush was made 6 h before a second crush, 1-1.5 cm proximal to the first crush 1–1[illegible] h after the second high crush the NA content of the isolated segment was assayed. The results indicated an increased NA content in the axonal granules of 75% already 3-4 h after the isolation of the segment, remaining constant up to 9 h after the second crush. The results indicate that axonal storage granules may increase their NA content by a factor of about [illegible] while being transported distally in the axons. This information together with the information from the preceding article of a mobile NA fraction of 45 [illegible] was used to calculate the rate of transport of NA granules proximal to a crush. The value obtained was 9 mm/h which is in good agreement with the value obtained for transport distal to a crush (8 mm/h) in the preceding article

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Acta physiol scand 1975 94 451–458

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On the Noradrenaline Loading in Axonal Amine Storage Granules in Rat Crushed Sciatic Nerves[1]

By

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Received 15 January 1975

Abstract

DAHLSTRÖM A J HÄGGENDAL and P A LARSSON *On the noradrenaline loading in axonal amine storage granules in rat crushed sciatic nerves* Acta physiol scand 1975 94 451–458

The increase of NA in rat sciatic nerve above a crush was investigated The transported amounts of NA did not increase quite linearly with time but more NA was found above the crush at 6 and 12 h than would be expected from the 3 h value One possible reason for this phenomenon— an increased NA loading of the accumulated axonal granules—was investigated by 2 types of double crush experiments One type involved simultaneous double crushes 1–1 5 cm apart The increase in NA in the isolated segment 6 h after crushing indicated that the axonal amine storage granules had increased their NA load by about 70 In the second type (delayed double crushes) a distal crush was made 6 h before a second crush 1 15 cm proximal to the first crush 1–12 h after the second high crush the NA content of the isolated segment was assayed The results indicated an increased NA content in the axonal granules of 75 already 3–4 h after the isolation of the segment, remaining constant up to 9 h after the second crush The results indicate that axonal storage granules may increase their NA content by a factor of about 2 (1 7) while being transported distally in the axons This information together with the information from the preceding article of a mobile NA fraction of 45 was used to calculate the rate of transport of NA granules proximal to a crush The value obtained was 9 mm h which is in good agreement with the value obtained for transport distal to a crush (8 mm/h) in the preceding article

The amine storage granules in peripheral adrenergic neurons are synthesized in the perikarya and transported distally in the axons to the nerve terminals (*cf* Dahlström and Häggendal 1966 Dahlström 1971) The granules are able to store the noradrenaline (NA) at an early stage and contain the NA during their axonal transport Therefore if a crush is applied to a nerve which carries adrenergic axons NA accumulates at a rapid rate proximal to a crush (Dahlström and Fuxe 1964 Dahlström 1965 Dahlström and Häggendal 1966) The protein components of the granules dopamine β hydroxylase (DβH) and chromogranin B also accumulate after crushing (Laduron and Belpaire 1968 Livett,

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Geffen and Rush 1968, Brimijoin 1972, Wooten and Coyle 1973). In parallel also dense core vesicles (presumably identical to amine storage granules) pile up in front of the constriction (Kapeller and Mayor 1967, Geffen and Ostberg 1969, Dahlstrom and Hokfelt 1971). Since dense core vesicles have been observed in perikarya and also in uncrushed axons (Hökfelt 1969, Geffen and Ostberg 1969), it is very likely that these storage organelles are—at least to a large extent—preformed in the cell body and transported distally in the axon as intact organelles.

NA or DβH in the axon have been used as "markers" for amine granules. The amounts of the two substances that accumulate above a crush during a certain period of time have been used to calculate the rate of transport of the amine granules. When the NA accumulation was used for this purpose (Dahlstrom and Häggendal 1966), the rate of transport in rat sciatic nerve was calculated to be in the order of 5 mm/h. For this calculation a number of assumptions were made: 1) *All* the NA in the sciatic nerve is transported distally in the axons at a fast rate, 2) The number of granules that are transported distally in the axons is constant with time at least up to 12 h, and 3) The granules contain a constant amount of stored NA—i.e. the granules do not increase their stores of NA while accumulated.

These assumptions have been reconsidered one by one during the last couple of years. In the preceding article (Häggendal *et al.* 1975) assumption no. 1 was studied and found to be incorrect, only about 50% of the NA in the sciatic nerve is mobile. Also assumption no. 2 may, under certain conditions, be non valid: the production and distal transport of granules is probably constant for up to 6 h, but thereafter in a cold environment the amounts of granules may increase (Dahlstrom and Haggendal 1972). The last assumption finally is dealt with in the present article. 3 types of experiments have been performed. At first a careful study was made to investigate whether or not, in rat sciatic nerve, the increase in NA content above a crush was unquestionably linear 3, 6 and 12 h after crushing. Secondly, simultaneous double crushes, 1–1.5 cm apart, were made, and the NA content in the separated nerve segment was measured between 0 and 9 h to see if the NA content was constant or increasing with time after separation. Thirdly, "delayed" double crushes were performed. In these experiments granules were allowed to accumulate for 6 h above a crush and then a second crush was made allowing the accumulated population of granules to alter their NA content uninfluenced by newly arriving granules. The results indicate that axonal amine storage granules do increase their load of NA by approximately 100% during accumulation and probably transport.

Material and Methods

Male rats of the Sprague Dawley strain (about '00 g) were used and kept at a room temperature of 3 C (±1 C) during the whole experiment. The sciatic nerves were operated bilaterally under ether anesthesia with 1 or 2 crushes a.m. Lubińska (1959).

In the first experiment a single crush was made 0, 3, 6 or 12 h before sacrifice. The 0.5 cm or 1 cm of nerve just proximal to the crush was dissected out and assayed for NA content according to Häggendal (1963). Two to 6 nerve segments were pooled for each estimation.

In the second experiment ("simultaneous double crushes") crushes were made at the same time 1.0–1.5 cm apart. After 0, 6 or 9 h, the rats were killed by a blow on the head, and the nerve segment between the crushes was dissected out. Particular care was taken that no material from the proximal

side of the high crush or distal to the low crush was contaminating the middle segment 4 to 8 nerves were pooled for estimation

The third experiment involved delayed double crushes, i.e. the 2 crushes were made at different times. The distal crush was always performed 6 h before the proximal crush. Zero, 1, 2, 3, 4, 6, 9 and 12 h after the second high crush the rats were killed and the segment between the crushes was dissected out. Generally 6 nerves were pooled for the NA estimation.

In every experimental series uncrushed nerves were included, pooled to a total length of 10–20 cm and assayed for NA content. Also 100 ng of NA was added to one or more samples to test the recovery of the estimation procedure (Häggendal 1963). The recovery was 83% as a mean and was corrected for.

Results

The results from the first experiment are shown in Fig. 1. The amount of NA which had been transported to the part of nerve just above the crush is indicated (solid line, Fig. 1). The transported amount was calculated by subtracting the NA amount in uncrushed or 3 h crushed nerve from the total amount of NA in the crushed nerve. It can be seen (Fig. 1) that the increase in transported NA is not linear. The observed 12 h value is higher than would be expected from the 3 h value (dotted line, Fig. 1).

In the simultaneous double crush experiments 2.21 ± 0.111 ng ($n = 16$) of NA was present per cm in the separated nerve segment in 0 h crushed nerves. At 6 h after crush the NA content per cm in this isolated segment had increased to 3.09 ± 0.215 ng ($n = 16$). As demonstrated in the previous paper (Häggendal *et al.* 1975) about 45% of the NA content in uncrushed nerves is rapidly transportable and probably located within axonal storage granules, in this case 45% of 2.21 ng = 0.995 ng. The rest 55% (= 1.216 ng) is presumably localized in nerve terminal granules of adrenergic nerve terminals innervating the blood vessels of the sciatic. It is known that nerve terminals increase their NA content by about 15% during the first 6 h after denervation (*cf.* Benmiloud and Euler 1963, Bareggi *et al.* 1975). Since our double crush procedure caused a denervation of the isolated segment, part of the increase observed was presumably confined to the nerve terminal portion of the sciatic NA. The increase in nerve terminal NA during the 6 h of crushing was thus

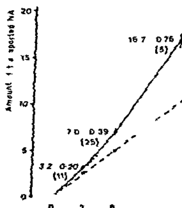


Fig. 1. The increase in noradrenaline (NA) in rat sciatic nerve above a crush is indicated (solid line). 3 or 10 mm segments just cranial to the crush were dissected out and assayed. The figures for NA increase with time after crushing were obtained by subtracting the NA content of 5 or 10 mm uncrushed nerve from the total NA content of the accumulated segment. Means ± S.E. are given; small numerals indicate number of observations. The dotted line indicates the theoretical NA increase with time had the increase proceeded as during the first 3 h period. The hatched line shows the theoretical curve for NA increase expected to be obtained in case the transported NA granules had an increased NA loading during transport and accumulation (correlation coefficient 0.98). For further explanation see text.

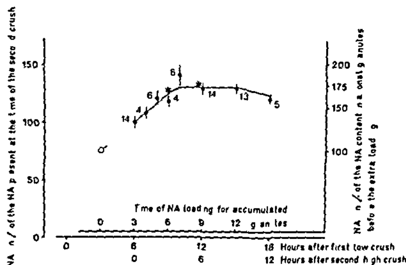


Fig . The increase in noradrenaline (NA) loading in isolated axonal granules of rat sciatic nerve Amine storage granules were allowed to accumulate above a low initial crush for 6 h At this time a second high crush was performed 1 1 5 cm proximal to the first crush The second crush prevented a further transport into the isolated segment of axonal granules from more proximal parts of the nerve 1 3 4 6 9 and 1. h after the second crush the segment was dissected out and assayed for NA content The dots ( ) represent mean ± S E (vertical bars) of the NA increase whi h can be attributed to increase in the NA loading of *axonal* granules after corrections for the ner e terminal NA (see text) Small figures represent number of observations

The hatched line indicates an extrapolation to 0 time of the extra loading of NA in the accumulated axonal granules

The two asterisks indicate the NA increase in axonal granules in the simultaneous double crush experiment.

15 % of 1 216 ng = 0 182 ng Of the total increase of 0 88 ng/cm only 0 70 ng (0 88 0 182 ng) therefore appeared to be connected with *axonal* storage granules The increase in *axonal* storage granules was thus from 100 % (45 % 2 21 = 0 99) to about 170 % (0 99 0 70 ng) When the 9 h values were used for similar calculations an increase of 173 % was obtained

The results from the delayed double crush experiment are shown in Fig 2 The stationary NA fraction (55% of the total NA in 0 h crushed nerves) was subtracted from the amount of NA present 6 h after the *first* crush *i e* at the time of the second high crush The values at 1 2 3 4 6 9 and 12 h after the *second* crush were likewise produced by subtracting the stationary NA in the nerve terminals (15 increase after denervation) from the assayed amount of NA in the separated segment between the two crushes It is clear from Fig 2 that the amount of NA in the axons of the separated nerve segment increased during the first 3-4 h after the second crush and then remained approximately stable during the next 8 9 h

Discussion

As shown in Fig 1 (solid line) the increase in NA proximal to a crush did not proceed absolutely linearly with time The increase per unit of time was larger at (to 9 and 9 to

12 h than at 0 to 3 h post crushing (compare solid and dotted lines) In the original par (Dahlstrom and Häggendal 1966) the increase was described as approximately linea both in cat and rat sciatic nerves Later investigators have found the increase to be linear in the 2.5 mm segment above crush in rat sciatic nerve (McLean and Keen 1972) and in the cat hypogastric nerve (Banks and Mayor 1972) The reason why these authors obtained a linear NA accumulation may be that the nerve segment was too short to include the whole extent of the NA accumulation (2.5 mm McLean and Keen 1972) or that the number of observations was too small (Banks and Mayor 1972) to disclose the rather small deviation from the straight line (Fig 1)

The reason for this deviation from the linearity which implies that more NA than expected was found in the segment proximal to the crush could be either a) that an increasing (with time) number of storage granules were transported distally in the axons and/or b) that the accumulated granules increased their NA content Alternative a) is unlikely because the temperature of the surrounding, which, if low can influence the synthesis of granules in the perikarya (Dahlstrom and Haggendal 1972) was kept constant at 23 C throughout the experiments Alternative b) is more likely because it is known that accumulated granules do synthetize NA (McLean and Keen 1972)

In order to examine further if accumulated granules can increase their NA load the simultaneous double crush experiment was done All the accumulated NA in double crushed nerves appears to be reserpine depletable (Dahlström 1967) the NA found in the segment 0 or 6 h after crushing is therefore most probably localized to storage granules Thus, since the isolated nerve segment contained a fixed number of axonal granules (no supply from more proximal segments because of the high crush) any increase in NA in this segment could be attributed to an increased NA content of the amine granules present in the segment The NA in the isolated segment increased from 2 21 ng/cm at 0 h to 3 09 ng/cm at 6 h. Only 45 % of the 0 h value (2 21 ng) was attributable to *axonal* storage granules (see Haggendal *et al* 1975) *i e* 0 995 ng The nerve terminal granules (containing 1 22 ng of NA) probably increased their NA content by about 15% after the denervation (cf Benmiloud and Euler 1963 Bareggi *et al* 1974) caused by the double crush procedure Therefore about 0 18 ng of the total increase in the isolated segment 6 h after operation was presumably confined to *nerve terminal* granules along blood vessels in the perineural connective tissue Thus 0 70 ng of the total increase of 0 88 ng presumably occurred within accumulated *axonal* granules These granules consequently appear to have increased their NA content from 0 99 ng to about 1 69 ng, *i e* by about 70

The delayed double crush experiments were performed to investigate if a larger amount of accumulated *axonal* granules (6 h of accumulation before interrupting further supply of granules by the second high crush) with higher initial content of NA would yield a more reliable result in terms of a clear increase As seen from Fig 2 the NA content in *axonal* granules in the isolated segment (total value corrected for nerve terminal NA at 0 h plus the denervation increase in nerve terminals at 1–12 h after the second crush) increased up to 3 h after the second crush and then remained rather constant up to 6 h At the time of the 2nd crush the *axonal* granules in the isolated segment had spent on the average 3 h in the segment, since some were arrested there just after the first crush, and other just

arrived to the segment 6 h later at the time of the second crush. The NA curve in Fig. 2 was therefore extrapolated to 3 h before the second crush. This point was considered to represent the NA amount in axonal granules before the onset of the extra loading (100% ordinate to the right in Fig. 2). The increase of NA in these granules after the extra loading would then be about 75%. The asterisks in Fig. 2 represent the corresponding values from the simultaneous double crush experiments. The increased NA loading in the *axonal* granules in the two experiments was thus very similar: 75%, and 70%, respectively. It is interesting that a similar increase in NA appear to occur in bovine splenic nerve granules during transport from proximal to distal segments (1.7 fold increase in the NA/protein ratio; Lagercrantz 1974).

These figures (about 75% increased NA content in accumulated *axonal* granules) may be used to calculate the theoretical accumulation curve for NA above a crush which should be observed were the NA content of the granules constant. This curve is indicated in Fig. 1 (hatched line) and was produced in the following way. Granules which were present above a 3 h crush had been accumulated for 1.5 h as a mean and had increased their NA content to about 117% (corresponding to 3.2 ng) from 100% (2.75 ng). Likewise, the 6 h value was corrected for an increase to 133% (7.0 ng) from 100% (5.26 ng). The 12 h value (16.7 ng) was corrected in the following way. If the number of granules which are transported to the segment during 12 h contain x ng of NA (indicating the relative number of granules) before the increased loading, then $0.5x$ granules have been present above the crush for 6 to 12 h. These granules have been maximally loaded from 100% to 175% (Fig. 2). The rest of the granules ($0.5x$) have been present in the segment for 0 to 6 h, *i.e.* 3 h as a mean, and have increased their NA from 100% to 133% (Fig. 2). Thus, the total number of granules after the extra loading contained $0.5x$ (175%/100%) + $0.5x$ (133%/100%) = 16.7 ng; x = 10.8 ng, which represents the original NA amount in the downtransported granules before the extra loading. The theoretical accumulation curve (hatched line, Fig. 1) obtained in this way is clearly different from the empirical curve (solid line, Fig. 1). According to the theoretical curve about 0.90 ng of NA would have been transported towards the crush region per hour. Since the transportable NA fraction in 1 cm of uncrushed nerve (no extra loading) contains 0.99 ng of NA, this constructed accumulation curve indicates a transport rate of about 9 mm/h. This is close to the figure for transport rate obtained in the previous paper on transport distal to a crush (about 8 mm/h; Häggendal *et al.* 1975). Also, 8 mm/h was found to be the rate of transport for a factor maintaining the nerve terminals (Häggendal *et al.* 1974; Bareggi *et al.* 1974).

When the D/H accumulation above a crush was used to calculate the rate of transport of amine granules in rat sciatic nerve, figures close to 8 mm/h were obtained (6.7.7 mm/h, Wooten and Coyle 1973; 9 mm/h, Häggendal, unpublished).

The increase in NA loading of the axonal amine granules found in this study probably represents an increase occurring in the granules during their normal transport distally in the axons. Their content of D/H is likely to remain constant during transport through the axons. Since the ratio of NA/D/H was found to be higher in distal segments of the sciatic nerve than in proximal parts (Häggendal, unpublished), this indicates an increased NA loading per granule during transport (see also Lagercrantz 1974).

3–4 h after the second crush the NA increase in the isolated segment levelled off (Fig 2) the NA content remaining rather constant up to 1–2 h after crush This may indicate that the granules have reached their maximal load by 3–4 h This is not in agreement with suggestions by other authors (cf Stjarne 1968 Lagercrantz 1971) who propose that nerve trunk vesicles (axonal granules) when reaching the nerve terminals may have increased their NA content by several orders of magnitude These suggestions are based on the large difference in NA/DβH ratio between axonal granules and nerve terminal granules However since DβH is lost from nerve terminal granules on repeated transmitter release while NA is resynthesized the majority of nerve terminal granules have a high NA/DβH ratio due to *a loss of DβH*—not necessarily due to a largely increased NA loading if compared to axonal granules The axonal granules have not yet been exposed to DβH loss at transmitter release and therefore contain less NA when related to DβH Stjärne (1968) has suggested that axonal granules increase their NA content more than 100 times when arriving in the nerve terminals on the basis of the NA/DβH ratio in the 2 populations of granules We conclude from the present results that axonal NA granules during their transport in the axons may increase their NA load (content) by a factor of 2 (see also Lagercrantz 1974) A further increased NA content per granule in the nerve terminals may be quite possible but it seems unlikely that *each granule* increases its NA content by 100 times

Supported by the Swedish Medical Research Counc l (projects 14X 2 07 o4P-4173 and 14X 166) and by Wilhelm & Martina Lundgren s Foundation The technical assistance of Mrs Elizabeth Ljung and Miss Mildred Pettersson is gratefully acknowled ed

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Acta physiol scand 1975 94 459–466
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Synthesis of Catecholamines in the Eye after Local Injection of ^{3}H-Precursors

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Received 17 January 1975

Abstract

RENTZHOG L. *Synthesis of catecholamines in the eye after local injection of ^{3}H precursors* Acta physiol scand 1975 *94* 459–466

The possib lity that adrenaline may be synthesized locally in the eye was investigated in the rabbit and vervet monkey L tyrosine 3 5 H L tyrosine (side-chain 2 3 ^{3}H) or L 3 4-dihydroxyphenylalanine (^{3}H(G)) was injected into the anterior chamber or the vitreous The animals were killed after 1/2 h up to 7 days in the tyrosine experiments and after 1 up to 4 h in the DOPA experiments. The synthesis of H adrenaline ^{3}H noradrenaline and ^{3}H-dopamine was measured with a modified version of a double isotope technique for determination of catecholamines No H adrenaline was found An attempt to stimulate the phenylethanolamine N methyltransferase activity with a glucocort coid and to inhibit the metabolism of catecholamines with a monoamino dase inhibitor also failed to demonstrate ^{3}H adrenaline synthesis ^{3}H noradrenaline was found in all experiments when H DOPA had been given The amounts were about 10 times higher after inject on into the anterior chamber than after i jection into the itreous H-dopamine was found in all except a few experiments after ^{3}H DOPA inject on The highest amounts were found after injection into the vitreous In experiments w th ^{3}H tyrosine we were unable to demonstrate any catecholamine synthesis This prompts the quest on of whether tyrosine hydroxylase may be absent in the eyes of certain animal species

Adrenaline in 1–2 solution is widely used as a pressure lowering drug in chronic simple glaucoma (Becker and Shaffer 1965) The action seems to be complex (Bill 1969 Kupfer *et al* 1971) Therefore it seemed of interest to investigate whether possibly adrenaline is formed locally in the eye as a local hormone or as a transmitter The present report deals with attempts to find adrenaline after local injection of precursors None was found but other interesting findings were made

Materials and Methods

Three catecholamine p ecursors were used L tyrosine (side-cha n 3-^{3}H) was obtained from the Radiochemical Centre Amersham England Its spec f c acti ity was 15 6 Ci mmol L tyrosine 3,5 H with a spec fic act ity of 48 C mmol and L 3 4-d hydroxyphenylalan ne-(^{3}H(G)) w th a spec fic act vity of 7.3 Ci mmol were obtained from New England Nuclear Corp Frankfurt/Ma n Germany The ra purity of each new batch wa checked by p per chromatography us ng n butanol acet ac d water (5 1

The purity was always greater than 95 [illegible] and the amount of DOPA in the tyrosine batches was always less than 1 [illegible] Preexperimentally 250 μCi of ^{3}H tyrosine or ^{3}H DOPA were given i.v. to 4 rabbits in order to check that ^{3}H adrenaline could be synthesized from the precursors used in the experiments. The rabbits were killed 1 h later and the amounts of ^{3}H adrenaline in the adrenals were analysed. ^{3}H adrenaline was found in all experiments. The ^{3}H activities bound to adrenaline were 1180 cpm and 1130 cpm per pair adrenals in the tyrosine treated animals, 1840 cpm and 1940 cpm in the DOPA treated animals. These values are in good agreement with those reported by Kvetňansky *et al.* 1971 in the rat.

For pretreatment were used fluoxyprednisolone 16,17 acetonide 40 mg/ml (Kenacort T® Squibb) and nialamide (Niamid®) Pfizer.

The experimental animals were albino rabbits with an average weight of about 2 kg and three vervet monkeys (*Cercopithecus aethiops*). Two of the monkeys used for steroid experiments had a weight of about [illegible] kg and the third 4.9 kg. The animals were divided in 6 different experimental groups.

I. Fifteen previously untreated rabbits were given L tyrosine (side-chain [illegible] 3 ^{3}H) 10 μCi/eye. The substance was injected with a fine syringe into the anterior chamber of one eye (9 animals) or into the vitreous (6 animals). *The injected volume was 10 µl/eye. The other eye served as an untreated control eye.* The rabbits were killed with an overdose of Nembutal® and the eyes were immediately enucleated. The experimental time before killing the animals was from 1/2 h up to 7 days. The injected eyes and the control eyes were pooled [illegible] and [illegible] for each analysis. One previously untreated monkey received 10 μCi/eye of the same substance into the anterior chamber of one eye and into the vitreous of the other. The monkey was killed after [illegible]4 h and each eye was analysed separately.

II. Three previously untreated rabbits were given L tyrosine 3,5-^{3}H 10 μCi into the anterior chamber of both eyes. The injected volume was 10 µl/eye. The animals were killed after 1 [illegible] and 4 h. One pair of eyes was used for each analysis.

III. Eight previously untreated rabbits received L 3,4-dihydroxyphenylalanine (^{3}H(G)) 5 μCi/eye into the anterior chamber of one of the eyes. This was given in a volume of 5 µl. The other eye served as an untreated control. Half of the animals were killed and enucleated after 1 h and half of them after 4 h. Two injected eyes were pooled for each analysis so that the total precursor amounted to 10 μCi per sample. The control eyes were also pooled [illegible] and [illegible].

IV. Seven rabbits were pretreated with Kenacort T 10 mg/kg b.wt. in a single dose i.m. 5 days before injection of 10 μCi ^{3}H DOPA in each eye. 5 of these rabbits got the injection into the anterior chamber and [illegible] of them into the vitreous. 4 rabbits were given Kenacort T and ^{3}H DOPA in the same doses and at the same time as above but [illegible] h before injection of the ^{3}H DOPA into the anterior chamber or the vitreous they received 100 mg/kg b.wt. of nialamide i.p. 4 rabbits got as the only pretreatment nialamide 100 mg/kg i.p. [illegible] h before injection of ^{3}H DOPA into the anterior chamber or the vitreous. The animals in this group were killed 1, 4 and [illegible]4 h after the injection of catecholamine precursor. One pair of eyes from each animal formed one sample in the further analyses.

V. Two monkeys were pretreated with Kenacort T 10 mg/kg b.wt. i.m. in a single dose 5 days and nialamide 100 mg/kg [illegible] h before the injection of the eye. One of these monkeys received [illegible]5 μCi ^{3}H DOPA and the other [illegible]5 μCi tyrosine 3,5-^{3}H into the anterior chamber of each eye. The animals were killed with Nembutal after 4 h. Each eye was analysed separately.

Analytical procedure

For the determination of [illegible]H adrenaline ([illegible]H A), [illegible]H noradrenaline ([illegible]H NA) and ^{3}H-dopamine ([illegible]H DA) in the eyes we used in a slightly modified version a double isotope derivative technique described by Rentzhog (19[illegible]). The original technique was developed for estimation of endogenous non radioactive catecholamines and a [illegible]H [illegible] is introduced in the catecholamine molecules by acetylation with [illegible]H acetic anhydride. In order to determine only the [illegible]H labeled catecholamines that had been synthesized from [illegible]H precursors, we [illegible] the acetylation with non labeled acetic anhydride. The different steps of the analytical procedure [illegible] in short (see Rentzhog 197[illegible] for detailed description).

A. The enucleated eyes were immediately [illegible]sected so that an "eye sample" consisted of the retina, the iris and the vitreous from [illegible] eyes. The eye samples were homogenized in 0.4 N perchloric acid containing [illegible] EDTA in an ice bath. Before homogenization 10 000 cpm of adrenaline 7 [illegible]C was added to each sample as internal standard.

B. Aluminium oxide passage with a batch procedure was used for primary isolation of the catecholamines.

C. After elution with formic acid [illegible] freeze-drying of the eluate the catecholamines were acetylated in an aqueous solution with non labeled acetic anhydride to give more stable derivatives.

TABLE I An example of the $^{3}H/^{14}C$ ratios after the different chromatographic steps and of the calculation of cpm ^{3}H initially bound to catecholamines synthesized from the ^{3}H precursors No ^{3}H was left in the adrenaline triacetate sample after the second chromatographic run (P4) indicating that no ^{3}H adrenaline had been synthesized The ^{3}H activities present as ^{3}H noradrenaline and ^{3}H dopaminetriacetate after the first chromatographic run (S1) could be calculated from the cpm ^{3}H in the final noradrenaline and dopaminetriacetate spots respectively and from the recovery of ^{14}C noradrenaline and ^{14}C-dopaminetriacetate in these spots The cpm ^{3}H initially bound to noradrenaline and dopamine were then calculated from the recovery of ^{14}C adrenaline after the first chromatographic run

Sample	Added ^{14}C-A cpm		$^{3}H/^{14}C$ ratio after chrom S1	recovery ^{14}C A after S1	Added ^{14}C NA triac or ^{14}C DA triac cpm	$^{3}H/^{14}C$ ratio after chromatography P4	S –S3	S5	S7	recovery ^{14}C NA triac or ^{14}C DA triac	Final ^{3}H cpm	Calculated ^{3}H cpm after S1	Calculated H cpm initially
IV 1	10 000	A	11 75	65		−0 06	−0 03				0	0	0
		NA			4 800	31 0	10 5	10 1	10 4	23	10 900	47 400	73 000
		DA			7 700	1 15	1 14			69	6 050	8 770	13 500

D Non labelled adrenaline noradrenaline and dopamine triacetates were added as carriers
E The triacetyl derivatives were extracted into chloroform and separated by silica gel TLC in system S1 An aliquot of the eluate from the adrenaline triacetate spot was counted by liquid scintillation The recovery of ^{14}C A after this chromatography passage was taken as a rough estimate of the losses also of NA and DA up to this analytical step

F As a standard in the following purification step a known amount of ^{14}C NA triacetate was added to the eluate from the NA triacetate spot and ^{14}C DA triacetate to the eluate from the DA triacetate spot. The A NA and DA triacetates were then rechromatographed separately in different solvent systems and small aliquots of the eluates were counted by liquid scintillation after each chromatographic run The chromatographic purification was continued until constant $^{3}H/^{14}C$ ratios were obtained indicating that pure substances had been isolated Second acetylation to tetraacetates of the NA triacetate and A triacetate was performed for further confirmation that pure catecholamines had been isolated

Chromatography systems listed in the order in which they were applied

S1 Chloroform acetic acid (– 1) Silica gel TLC
P4 Toluene ethylacetate methanol water (10 1 5 5) (organic phase)
Paper chromatography (Whatman No 1)
S S3 Chloroform methanol (9 1) chloroform acetic acid (1 1)
Two dimention l silica gel TLC
S5 Chloroform cyclohexane acetic acid methanol (5 3 1 1) Silica gel TLC This system was only used in those cases where the $^{3}H/^{14}C$ ratios were not constant between P4 and S2–S3
S8 Chloroform acetic acid (4 1) Silica gel TLC This system was used for the tetraacetylated catecholamines

G Calculation The amounts of ^{3}H A in the eyes were calculated from the cpm ^{3}H and the recovery of ^{14}C A standard after the last chromatography The amounts of ^{3}H NA and ^{3}H DA in the tissues were calculated from the cpm ^{3}H and the recovery of ^{14}C NA triacetate/^{14}C DA triacetate after the final chromatography and from the recovery of ^{14}C A after the first chromatographic run An example of the calculation is shown in Table I

Results

In experimental group I where previously untreated rabbits and one monkey were given about 6 6 10− mol of L tyrosine (side-chain 2 3 ^{3}H) into the anterior chamber or vitreous we could not detect any synthesis of A NA or DA from the radioactive p

TABLE II ^{3}H activities calculated to be present as ^{3}H noradrenaline in one eye after local injection of ^{3}H DOPA Each value corresponds to one analysis (Two pooled or one separately analysed eye) The values are given as percentage of the injected ^{3}H DOPA activity and as mol × 10 ^{12} NA *Different amounts of ^{3}H DOPA were given in the 3 experimental groups*

Exp group	Species	Pretreatment	Inj into the anterior chamber (exp time h)						Inj into the vitreous (exp time h)			
			1		4		24		1		4	
				10^{-12} mol		10^{-12} mol		10^{-12} mol		10^{-12} mol	%	10^{-12} mol
III	Rabbit	None	1 17 0 37	8 7 2 6	0 88 0 40	6 3 2 8						
IV	Rabbit	Kenacort T	0 67 0 74	9 4 10 4	0 79 1 40	11 0 19 6	0 11	1 5	0 09	1 3	0 06	0 8
IV	Rabbit	Kenacort T+ nialamide	0 43	6 0	0 14	2 0			0 03	0 4	0 01	0 1
IV	Rabbit	Nialamide	0 28	3 9	0 21	2 9			0 03	0 4	0 0	0 3
V	Monkey	Kenacort T nialamide			1 01 1 09	35 38						

Similar findings were made in experimental group II where previously untreated rabbits got about 2 2 × 10 ^{10} mol of L tyrosine 3 5 ^{3}H into the anterior chamber The lowest over all recoveries in the analysis were 10% for NA and A and 30% for DA This means that the lowest detectable amounts of ^{3}H A and ^{3}H NA were 6 6 × 10 ^{14} mol in group I and 2 1 × 10 ^{14} *mol in group II The values for ^{3}H DA were 2 2 × 10 ^{14} mol and 0 7 × 10 ^{14} mol* respectively This corresponds for A and NA to 0 01% and for DA to 0 003% of given amount of precursor

In group III previously untreated rabbits were given about 7 × 10 ^{10} mol of ^{3}H DOPA into the anterior chamber of one eye and no treatment in the other eye No ^{3}H A or ^{3}H DA was found in these samples but synthesis of ^{3}H NA was seen The amounts of ^{3}H activity bound to NA in the tissues were calculated to 1 17% and 0 37% of the injected ^{3}H activity in the two rabbits killed 1 h after the injection The figures for the two animals killed 4 h after the injection were 0 88% and 0 40% (see top line Table II) These figures correspond to 8 2 × 10 ^{13} mol and 2 6 × 10 mol (1 h) and 6 3 × 10 ^{13} mol and 2 8 × 10 mol of NA (4 h) from the injected amount 7 × 10 mol of DOPA Small amounts of tritium were lost *from the generally labelled ^{3}H DOPA during the synthesis and these figures therefore are* slightly underestimated No ^{3}H NA was found in the control eyes The lowest detectable amount of ^{3}H A and ^{3}H NA in this group was 14 × 10 ^{14} mol and of ^{3}H DA 4 × 10 ^{14} mol

The rabbits in group IV were pretreated with glucocorticoid glucocorticoid MAO inhibitor or only MAO inhibitor before injection of about 14 × 10 mol of ^{3}H DOPA into the anterior chamber or the vitreous No ^{3}H A was found in these samples All samples contained both ^{3}H NA and all except one ^{3}H DA Table II shows the ^{3}H activity calculated to be present as ^{3}H NA and Table III the ^{3}H DA values. The highest amounts of ^{3}H NA were found 1 and 4 h after DOPA injection into the anterior chamber and after pretreat

TABLE III ^{3}H activities calculated to be present as ^{3}H-dopamine in one eye af er local injection o DOPA. See text Table II

Exp group	Species	Pretreatment	Inj into the anterior chamber (exp time h)						Inj into the vit eous (ex tim r			
			1		4		24		1		4	
				10^{-12} mol		10^{-12} mol		10^{-12} mol		10 mol		10^{-12} ol
III	Rabbit	None	<0 003		<0 003							
IV	Rabbit	Kenacort T	0 11 0 13	1 5 1 8	0 04 0 06	0 6 0 8	<0 003		0 83	11 6	0 25	3
IV	Rabbit	Kenacort T+ nialamide	0 11	1 5	0 17	2 4			0 90	1 6	1 30	18 2
IV	Rabbit	Nialamide	0 20	2 8	0 14	2 0			0 47	6 6	1 31	18 5
V	Monkey	Kenacort T+ nialamide			0 007 0 004	0 0_4 0 014						

ment with Kenacort T These values however were about equal to those found for ^{3}H NA in the untreated group III Injection of ^{3}H DOPA into the vitreous gave about 10 times lower H NA values than if precursor was given into the anterior chamber

In contrast to the findings with NA the highest values of ^{3}H DA were found after injection into the vitreous Lower values were noted after injection into the anterior chamber There was no difference in ^{3}H DA values between the 3 different pretreatments (see Table III)

Group V consisted of 2 monkeys pretreated with glucocorticoid and MAO inhibitor One of them was given about 5 5 × 10^{-10} mol of tyrosine 3 5 H and the other one about 35 × 10^{-} mol of ^{3}H DOPA into the anterior chamber of each eye Similarly to the rabbit find ings we found no synthesis of any ^{3}H-catecholamine after tyrosine H DOPA gave no ^{3}H A but both ^{3}H NA and H DA were found (see Table II and III bottom line) The H NA values agreed reasonably well with those in rabbits but there was much less H DA

Discussion

In a previous report (Rentzhog 1972) the difficulties of identifying and measuring small quantities of one catecholamine in the presence of high concentrations of others in a tissue were discussed The same problem arises in studies of catecholamine synthesis from radioactive precursors where several metabolites—some of them perhaps unknown—and high concentrations of other catecholamines may obscure the synthesis of a number of cate chols in low concentrations The radioactive substances formed in precursor experiments are usually separated by column and/or thin layer chromatography Roberts (1966) and Forrest *et al* (1970) have shown that chromatography of catecholamines may also display false double spots with Rf values close to those of other catecholamines It is desirable convert the catecholamines to more stable derivatives so that a careful separation w

veral different purification procedures may take place to allow detection of catecholamines which have been synthesized in very small quantities Rentzhog (1972) has described a double isotope method for assaying endogenous catecholamines With this technique the catecholamines are acetylated so as to obtain more stable and easily separated derivatives In the original method this was done with radioactively labelled acetic anhydride In the present study cold acetic anhydride was used instead and the double isotope ratio necessary for following the purification procedure was obtained from the ratio between the ^{3}H activity of the substances formed from the ^{3}H precursors and the ^{14}C activity from added standard catecholamines A constant ^{3}H/^{14}C ratio in successive chromatographies in different systems indicates that a pure catecholamine has been isolated The finding that the ^{3}H/^{14}C ratio was unaltered after reacetylation (see Rentzhog 1972) was regarded as further evidence of purity From the recovery of ^{14}C standards the losses of ^{3}H catecholamines during the purification procedure could be calculated and compensated for An example of the ^{3}H/^{14}C ratio after the different steps of purification and of the calculation of recoveries are presented in Table I

The possibility that adrenaline may be synthesized outside the adrenal glands and perhaps serve as a transmitter has been discussed (Rentzhog 1972) Several investigators have demonstrated adrenaline in rat brain by biochemical methods (Vogt 1954 Gunne 1962 Rentzhog 1972) Experiments by Ciaranello *et al* (1969) Pohorekey *et al* (1969) and Koslow and Schlumpf (1974) also appeared to show that enzymatic synthesis of adrenaline took place in the brain of several animal species Hokfelt *et al* (1973) found that certain neurons in the reticular formation of the rat contained an enzyme which produced antibodies as an immunological response to adrenal phenylethanolamine N methyltransferase (PNMT) Saavedra *et al* 1974 have given further evidence for the localisation of PNMT in several rat brain nuclei L Hermite (1971) gave systemic injections of ^{3}H tyrosine and ^{3}H DOPA to rats and found adrenaline in the iris and ductus deferens Unfortunately he did not test adrenalectomized animals and the possibility that the adrenaline found in the eyes was taken up from the blood stream cannot be excluded In our experiments we therefore considered it important to give the precursors in local injections Neither in the rabbit nor in the monkey however did we find any evidence of adrenaline synthesis in the eye

Wurtman and Axelrod (1965) have shown that the PNMT activity in the adrenals is controlled by cortisol and corticosterone secreted by the adrenal cortex It might be possible therefore that circulating corticosteroids may also influence adrenaline synthesis if such takes place in peripheral neurons We were unsuccessful however in demonstrating any adrenaline synthesis in the eye following pretreatment with a high dose of steroid An attempt with inhibition of the metabolism of the newly formed catecholamines with a monoamino oxidase inhibitor also failed to demonstrate adrenaline synthesis

When ^{3}H DOPA was injected as a precursor both into the anterior chamber of the eye and into the vitreous synthesis of ^{3}H noradrenaline was obtained as expected About 10 times higher quantities of ^{3}H NA were measured after injection into the anterior chamber The reverse was found for the synthesis of ^{3}H-dopamine In fact in 2 expts on untreated rabbits no ^{3}H DA was detected 1 and 4 h after injection into the anterior chamber Following pretreatment both with glucocorticoid and with nialamide however ^{3}H DA synthesis

was established even on injection into the anterior chamber The greater formation of DA following injection into the vitreous and of NA following injection into the anterior chamber is well in accord with the distribution of catecholaminergic neurons The retina seems to be rich in dopaminergic cells (Haggendal 1965) while the iris is rich in noradrenergic nerve endings.

A surprising result of our studies was that when ^3H tyrosine was given as precursor no synthesis of either ^3H DA or ^3H NA was established in either animal species The possibility that our ^3H tyrosine was inactivated was excluded by the finding in rabbit experiments that ^3H tyrosine given i v resulted in formation of ^3H adrenaline in the adrenals This prompts the question of whether tyrosine hydroxylase (TH) may be absent in the eyes of certain animal species In a series of preliminary experiments Palmberg and Podos (personal communication 1972) found that there was no detectable TH in an iris-ciliary body preparation from albino rabbits or brown Hartley guinea pigs Pretreatment with reserpine that boosted the rabbit adrenal TH activity 15 fold resulted in no TH activity in the eye preparation

Another possible explanation for our results is that tyrosine is involved to a greater extent in other metabolic pathways in the eye than in other tissues This would mean that too little tyrosine would be available for the catecholamine synthesis It is also possible that the quantities of tyrosine administered in our experiments were too low as injected tyrosine will be diluted by tyrosine present endogenously Reddy *et al* (1961) found that the tyrosine concentration in the aqueous humour was 1×10^{-4} mol per litre This means that in an eye the amount of tyrosine in the aqueous humour may be expected to be about $2\ 5 \times 10^{-8}$ mol The radioactively labelled tyrosine injected into the anterior chamber will therefore be greatly diluted by endogenous tyrosine resulting in a reduction of the specific activity A dilution with the amounts of endogenous tyrosine mentioned above would reduce the lowest detectable dose of ^3H NA in the eye to about 2 5 10^{-} mol in the rabbit experiments and to about 2×10^{-12} mol in the experiments on monkeys These figures may be compared with the quantities of ^3H NA found in our studies following injection of ^3H DOPA into the anterior chamber in the rabbit 2 0–10 6 × 10 mol and in the monkey 35–38 10^{-12} mol

Our experiments have not shown with certainty that in these animals catecholamine can not be synthesized in the eye from tyrosine but on the basis of these findings it would seem of value to study further the sources of catecholamine synthesis in the eye of the monkey and other animals

The skilful techn cal assistance of Mrs Laine Benn ch Björkman is gratefully acknowledged The work was supported by grant EY 00231-09 from the National Eye Institute US PHS to Professor E Bárány and grant B74-04X 733-09C from the Swedish Med cal Research Council.

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Acta physiol scand 1975 94 467–471
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Renal Handling of Human β_2-Microglobulin in the Rat The Importance of Sham Operation

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Received 23 January 1975

Abstract

RAVNSKOV U and A KARÁTSON *Renal handling of human β_2 microglobulin in the rat The importance of sham operation* Acta physiol scand 1975 *94* 467–471

The plasma disappearance rates of human β_2 microglobulin and (hydroxy methyl C^{14})-inulin was measured in unoperated sham-operated ureter ligated and nephrectomised rats Judging from the C^{14} activity curves in ureter ligated and nephrectomised rats a substantial glomerular filtration continued after obstruction of the urine flow Almost 90 % of the injected protein had disappeared from plasma 120 min after injection into nephrectomised rats indicating a considerable extra renal elimination and invalidating attempts to evaluate different renal mechanisms of elimination before and after ureter ligation Sham operation induced an increase of the plasma disappearance of both β_2 microglobulin and inulin The latter phenomenon may explain earlier findings of similarity between the disappearance curves of low molecular weight proteins in unoperated and ureter ligated animals and stresses the importance of using sham operated animals as controls

The elimination of low molecular weight (LMW) proteins is mainly renal This has been demonstrated by measurements of the disappearance rate of Bence Jones proteins and L chains (Wochner Strober and Waldmann 1967 Mogielnicki Waldmann and Strober 1971) parathyroid hormone (Martin Melick and de Luise 1967) and β_2-microglobulin (Bernier and Conrad 1969) in laboratory animals Such studies have shown that the disappearance rate of these proteins is similar in ureter ligated animals and unoperated controls, while it is considerably prolonged after nephrectomy Most authors who have used this approach have suggested a mechanism of glomerular filtration of the protein followed by proximal tubular reabsorption and catabolism They have explained the unaltered elimination rate of the LMW proteins after ureteral ligation by the filtration and reabsorption continuing uninterrupted after obstruction of the urinary flow The degree of glomerular filtration after ureteral ligation was not measured in these studies however Neither was the influence of sham-operation investigated

Measurements of the renal extraction rate of LMW proteins were additional evidence of renal elimination of these proteins Such studies have shown that the extraction rate of insulin in dogs (Zaharko Beck and Blankenbaker 1966) and humans (Chamberlain and

Stimmler 1967 Rabkin *et al* 1970) exceeded the filtration fraction and the relation between the extraction rate of β_2-microglobulin and inulin was found to be correlated with the extraction rate of p-amino-hippurate (Ravnskov Johansson and Göthlin 1972) These findings suggest a direct renal uptake of LMW proteins in addition to glomerular filtration and proximal tubular reabsorption Such an uptake independent of the glomerular filtration rate and urinary flow could seemingly explain the high elimination rate of LMW protein in ureter ligated laboratory animals

To explain the differences between the disappearance rates of LMW protein in normal and ureter ligated animals simultaneous measurements of the elimination of a substance known to be eliminated by glomerular filtration only was investigated Since previous workers have used unoperated animals as controls the effect of sham-operation was studied also

Methods

Experimental approach

Human β_2-microglobulin and C^{14} inulin were injected i v into 5 normal ("unoperated) 6 sham-operated 7 ureter ligated, and 5 nephrectomised rats and the plasma level was measured from 3 to 1 0 min after administration

Procedure

Experiments were performed on male Wistar rats weighing .50–400 g. They were allowed water *ad lib* and anesthetised with sodium pentobarbital (4 mg/100 g b wt.) injected into the abdominal cavity As a rule light ether anesthesia was added during the last 30–45 min of each experiment The left jugular vein and carotid artery were ligated cranially and a polyethylene catheter (PE 60 Clay Adams New York) was placed in the caudal parts of the vessels The kidneys and ureters were exposed transabdominally through a *mid l ne incision except in the unoperated group*

Ureter l gation Ureters were ligated bilaterally at two places but not divided Completeness of l gation was controlled by counting the C^{14} acti ity of the urine drawn from the bladder after the cessat on of each experiment Rats with urine activity greater than the activity of plasma drawn 60 min after the injection were d scarded

Neph ectomy was performed by ligating and by cutting of the renal pedicle

Sham-ope ation included exposure of the kidneys and digital palpation of the ureters

Sampl ng 0 1–0 . ml of a 1 solution of human β_2 microglobulin in an 0 01 M phosphate buffer pH 7 3 and 0 1–0 . ml saline containing 10 µCi (hydroxy methyl-C^{14})-inulin per ml were injected via the indwelling jugular catheter The non nephrectomised rats were generally injected by more β_2-microglobul n than the nephrectomised to achieve measurable amounts in the plasma at the 1?0 min point. Blood samples of 0 3 ml were drawn *via* the catheter placed in the carotid artery 3 6 15 30 60 and 1?0 min after administration. After each sampling the catheter was washed with 0 –0 3 ml sal ne contain ng 5 IU heparin per ml D sposable needles and PVC syringes were used for the blood sampl ng

Substances

Human β_2-microglobul n was isolated from the urine of patients with severe uremia The ur ne was concentrated against solid polyethylene glycol (mol. wt 1 000) using .3 3 inch dialysi g tub ng (Union Carbide) and the proteins were separated by an on exchange chromatography in a 60 0 9 cm column containing Whatman DE 5. equ l brated in an 0 01 M Tris HCl buffer pH 7 85 with a g adient of 0 01 to 0 1 M NaCl The β_2-microglobul n-rich fractions were pooled concentrated and further sep ra ed by chromatography on Sephadex G 75 superf ne equ l brated in an 0 01 M Tris HCl buffer pH 7 3 containing 0 ? M NaCl The fractions containing β_2-microglobul n were d lysed against re i ed water and lyophil sed.

(*Hydroxy-methyl C^{14})-inul n* a substance known to be a e at nat e i ul n in the rat (M row and Sheppard 1970) was supplied from the Radiochemical Centre Amers am England Accor g to the

TABLE I Concentration of human β microglobulin and C^{14} activity (mean ±S E) in plasma samples drawn at different time intervals after intravenous administration of the protein and (hydroxy methyl C^{14})-inulin

		3 min (mg/l) (cpm)	6 min (of value at 3 min)	15 min (of value at 3 min)	30 min (of value at 3 min)	60 m n (of value at 3 min)	1 0 min (of value at 3 min)
Unoperated controls (n=5)	β microglobulin	44 7±13 8	63 3±2 5	26 0±2 2	10 4±1 4	5 2±0 8	2 3±0 4
	C^1 activity	5 689±1 031	71 2±5 1	43 5±2 .	32 1±2 6	21 0±2 2	14 7±5 5
Sham operated (n=6)	β microglobulin	36 8±5 2	62 6±3 3	19 2±3 3	5 2±1 3	2 3±0 6	1 0±0 3
	C activity	5 935±939	65 1±3 9	30 5±3 0	16 0±3 1	9 1±1 8	4 4±1 1
Ureter ligated (n=7)	β_2 microglobulin	31 7±9 5	64 9±4 2	27 1±4 5	11 6±2 2	5 4±1 2	3 .±0 7
	C^{14} activity	5 263±827	63 6±2 9	40 0±3 5	9 3±3 4	.1 3±3 0	17 5±2 8
Nephrectomised (n=5)	β_2-microglobulin	23 9±4 1	71 9±3 7	44 8±4 4	26 5±5 1	18 0±3 5	11 6±3 4
	C 4 activity	11 627±3 042	67 2±5 2	49 6±5 2	4? 6±5 2	38 8±5 3	36 4±6 1

manufacturer the specific activity was 1 78 μCi/mg the average molecular weight approx mately 5 200 and the radiochemical purity 99

An lyses

β microglobulin was determined in duplicate by radial immunodiffusion as described previously (Ravn skov 1973) C^{14} activity in plasma and urine was counted in a Nuclear Chicago Mark 2 liquid scintillation counter Before counting dupl cate samples of 30 μl were solubilised in Soluene® (Packard Ill U S A) The level of β_2 microglobulin and the C^{14} activity were calculated in percentage of the value obtained n the plasma drawn 3 min after the injection

Results

The decrease of the plasma level of β_2 microglobulin was considerably more rapid than that of C^{14} inulin in all groups (Table I and Fig 1) including the nephrectomised rats Thus the plasma half tim after nephrectomy calculated from the slope of the disappearance curves between 60 and 120 min was 103 min for β microglobulin but 485 min for C inulin Expressed in another way in comparison with the 3 min values 88 4 of β_2 microglobulin but only 43 6 of C inulin had disappeared from plasma at 120 min

The disappearance curves in ureter ligated and unoperated controls did not differ significantly This was evident for both C inulin and β microglobulin However there was a highly significant difference between ureter ligated and sham operated rats Accordingly there was a great difference between the disappearance curves in unoperated controls and in sham operated animals Also this difference was of a similar magnitude for β_2 microglobulin and C^{14} inulin

Discussion

The considerable delay of the plasma disappearance rate of β_2-microglobulin after nephrectomy stresses the importance of the kidney in the elimination of LMW protein The

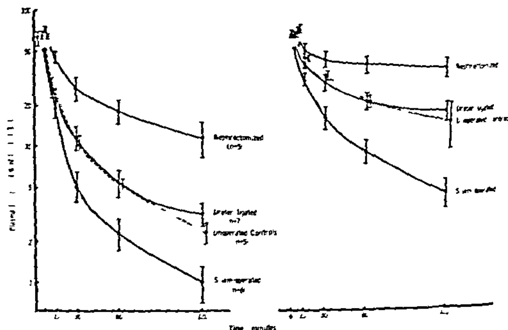


Fig. 1 Plasma disappearance curves of human β_2-microglobulin (left) and C^{14} inulin (right) in nephrectomised and ureter-ligated rats, and in sham-operated and unoperated controls. Bars indicate ±1 S.E. The values are given in Table I

ference between the disappearance curves of inulin and β_2-microglobulin demonstrates that a substantial extra-renal mechanism of elimination also exists. Similar findings were presented by Bernier and Conrad (1969)

The elimination of β_2-microglobulin was less affected by ureter ligation than the elimination of inulin, apparently pointing to a direct renal uptake of β_2-microglobulin in addition to glomerular filtration, but the difference was small and may as well be explained in the following way. Judging from the differences between the disappearance curves of inulin in ureter ligated and nephrectomised rats glomerular filtration does not cease completely after ureteral ligation. Consequently ultrafiltrate accumulates in the tubules. Due to tubular reabsorption of water and β_2-microglobulin, filtration of inulin after ureteral occlusion will occur against an increasing concentration gradient, whereas no or only a minor gradient will oppose the filtration of β_2-microglobulin.

Earlier observations demonstrating an unchanged disappearance curve of LMW proteins after ureter ligation (Wochner, Strober and Waldmann 1967; Mogielnicki, Waldmann and Strober 1971; Bernier and Conrad 1969) was the stimulus to the present work. In these previous studies of the renal catabolism of proteins, ureter ligated and nephrectomised animals were compared with unoperated animals. The great difference between the disappearance rates in unoperated and sham-operated animals observed in the present study explains the high disappearance rate after ureter ligation in the mentioned works. The difference between the disappearance curves of inulin in ureter ligated and sham-operated animals in our study is also consistent with the results of Salomon and Lanza (19[illegible]) who

found that ureter ligation induced a substantial reduction but not a total disappearance of the renal extraction rate of inulin

Half time calculations showed that the discrepancy between unoperated and sham operated rats was confined mainly to the early part of the experiments suggesting that the explanation may be an increased distribution volume in the sham-operated animals One cause could be the formation of a traumatic edema a well known consequence of surgery (Moore *et al* 1963)

An overestimation of the elimination rate of a protein injected into abdominally operated animals may thus be introduced Since the inulin disappearance was also increased after surgery the phenomenon is likely to be valid for any substance at least if of a similar molecular size and it stresses the importance of using sham-operated controls in such studies. Accordingly a re-evaluation of earlier data on protein elimination is needed Previous findings of similar disappearance curves in unoperated and ureter ligated animals cannot be taken as evidence of a direct renal uptake This mechanism has not been excluded by the present study but its existence should be demonstrated by other methods

This study was supported by a grant from the Medical Faculty of the University of Lund and from the Swedish Medical Research Council (grant no 19 X-4542) We are indebted to Ms Ilga Priediti for skillful technical assistance

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Acta physiol scand 1975 94 472–483
From the Institute for Experimental Medicine University of Copenhagen Denmark

The Effect on Total Renal and Tubular Function and Plasma Renin of a Moderate Isotonic Saline Load in Rats Anesthetized with Amytal and Inactin

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Received 31 January 1975

Abstract

LEYSSAC P P O FREDERIKSEN and S L SKINNER *The effects on total renal and tubular function and plasma renin of a moderate isotonic saline load in rats anesthetized with Amytal and Inactin* Acta physiol scand 1975 94 472–483

The renal effects of i v saline loading equal to 1 body weight (b wt) were studied in groups of rats *group I* was anesthetized with Amytal (15 mg/100 g b wt plus supplementary doses) *group II* with Inactin (12 5 15 0 mg/100 g b wt.) *In group I* the saline load caused an increase in urine flow (+9) sodium excretion (+67) inulin clearance (C_{IN}) (+ 4) PAH clearance (+31) and absolute proximal reabsorption rate (+27) Proximal fractional reabsorption and filtration fraction (FF) remained unchanged while plasma oncotic pressure (COP) decreased by 10 Plasma renin fell and there was an inverse relationship between renin concentration and proximal reabsorption rate In *group II* urine flow and sodium excretion increased after saline (+85 and 110 respectively) C_{IN} and absolute proximal reabsorption rate was lower than in group I and failed to increase after saline Proximal fractional reabsorption was also lower and decreased after saline COP as well as renin decreased as in group I but no relationship between renin concentration and proximal reabsorption rate could be demonstrated The data indicate that Inactin depresses both resting proximal tubular reabsorptive capacity and the tubular response to a physiological volume expansion The results are compatible with the hypothesis that the renin angiotensin system is significantly involved in regulation of proximal tubular function while they are incompatible with the idea that peritubular COP plays any major role in this adjustment

It has long been recognized that the administration of isotonic saline increases the glomerular filtration rate (GFR) in the dog without change in filtration fraction (FF) (Smith 1951) and that the accompanying rise in the rate of excretion of water and solutes is smaller than the change in their rates of filtration This observation indicated that tubular reabsorption rate of solute and water must have increased during the rise in GFR with saline administration However later micropuncture studies have consistently demonstrated in the dog that isotonic volume expansion to 4 of body weight (b wt) depresses not only fractional but also absolute reabsorption rate by the proximal tubule (Dirks *et al* 1965 Davis *et al* 1970

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Mandin *et al* 1971 Herrera Acosta *et al* 1972 Wen *et al* 1973) In the rat most investigators have drawn the same conclusion in micropuncture studies with isotonic volume expansion to about 10% of b wt (Bank *et al* 1969 Brenner *et al* 1971 Daugharty *et al* 1972 Bartoly and Earley 1972 Stein *et al* 1973) More moderate isotonic saline loading has also been reported to depress absolute proximal reabsorption rate in the rat (Davidman *et al* 1972 Brenner and Berliner 1969)

These and similar observations have apparently given the impression that isotonic saline loading invariably depresses proximal reabsorption rate also in the rat The results are further taken as indirect support for and are generally interpreted according to the hypothesis of a key role of peritubular colloid osmotic pressure (COP) in regulating proximal tubular reabsorption (Earley *et al* 1966 Windhager *et al* 1969 Brenner *et al* 1969 1971)

However all these results in rats have been obtained in animals anesthetized with the thio-barbiturate Inactin In contrast to the oxy barbiturate Amytal anesthetic doses of Inactin have been shown to inhibit net fluid transport by proximal tubules *in vivo* (Elmer *et al* 1972) and isosmotic fluid transport by rabbit gall bladder *in vitro* (Christensen *et al* 1973) Furthermore Steven (1974 a) has recently reported that the proximal tubule wall is far less resistant to transmural pressure changes (is more collapsable) in animals anesthetized with Inactin as compared with Amytal anesthetized rats It is therefore of importance that Cortney *et al* (1965) studying rats anesthetized with the oxy barbiturate pentobarbital failed to demonstrate any inhibition of absolute proximal reabsorption rate even after extreme isotonic saline loadings to 10% of b wt Because the degree of volume expansion and both the dose and the particular anesthetic used could have contributed to these various findings the renal effects of more moderate isotonic saline loading (1% b wt) were investigated in two parallel series of rats the one anesthetized with Amytal and the other with Inactin The data show that in Amytal anesthetized rats moderate isotonic volume expansion significantly augments not only urine flow and solute excretion but also GFR and proximal reabsorption rate without changing FF With Inactin anesthesia GFR and proximal reabsorption rate fail to increase with the rise in urine flow and solute excretion rate Furthermore plasma COP was depressed by saline loading in both series Thus the data are incompatible with the peritubular COP as a major determinant of the proximal fluid reabsorptive capacity in normally hydrated and moderately saline loaded rats They also indicate that previous conclusions concerning the effect of saline loading on proximal fluid reabsorption (may) have been misleading due to the use of an anesthetic which itself depresses this process On the other hand the data are consistent with the hypothesis that the renin angiotensin system plays a role in regulating proximal reabsorption rate in rats anesthetized with Amytal

Methods

Male SPF Sprague Dawley rats weighing about 250 g were allowed free access to food and water prior to the experiment. Anesthesia was introduced and maintained by intraperitoneal (i p) injections of barbiturate

Group I was given sodium amobarbital (sodium Amytal) 15 mg/100 g b wt and anesthesia was supplemented when necessary with usually 2 and at most 3 additional i p doses of 1 2 mg/100 g b wt

Group II was given Inactin (Promonta Hamburg) Animals were selected excluding all rats which would not sleep sufficiently well for operation within 0 min at a maximum dose of 15 mg/100 g b wt The total dose administered ranged from 1 5 to 15 mg/100 g b wt

Rats were placed on a servo-controlled heated micropuncture table maintaining body tempera ure at about 37 C, and prepared for clearance proximal occlusion time (OT) and transit time (TT) measurements as previously described (Leyssac 1964 Bojesen and Leyssac 1969) Polyethylene catheters were inserted into the left jugular vein for infusions and into the right carotid artery for blood sampling and continuous recording of systemic arterial pressure (BP) with a capacitance pressure transducer (Hansen S & W) and Servogor recorder

A priming dose of 30 mg para amino-hippurate (PAH) and/or 25–30 mg polyfructosan (Inutest, Laevosan) in saline was given i v and followed by a continuous infusion of 1 2 mg PAH and/or 0 3 mg polyfructosan pr min in a volume of 15 μl/min throughout the experiment Experiments started after an equilibration period of about 45 min

After collection of the first blood sample (about 200 μl) 2 to 4 serial urine samples were collected into calibrated polyethylene tubing within 10–30 min for clearance determinations OT was measured immediately after the last urine collection as previously described (Leyssac 1964) 2–3 min later when urine flow and BP had reached the preceding level 75–100 μl 5 Lissamine green in 0 9 saline at pH 7 4 were injected i v and TT measured by the modification of Steinhausen s method (1963) as described by Gertz *et al* (1965) Blood samples (200 μl) from the renal vein and the carotid artery were collected immediately after the TT measurements

After the first control clearance period 2 5 ml 0 9 saline were given i v at a rate of 1 0 ml min and followed by a continuous infusion of 15 μl/min of isotonic saline 30 min later a second clearance period was started including an initial blood sample 3–4 serial urine collections OT and TT measurements and final blood samples from the renal vein and the carotid artery

After the experiment the left kidney was removed drained and weighed Inulin in urine and plasma was measured by the diphenyl amine method of Bojesen (1952) modified for microanalysis. Osmolarity of plasma and urine was measured with the cryoscopic method of Ramsay and Brown (1955) PAH in urine and plasma was measured by the method of Smith *et al* (1945)

Intratubular pressures were measured by the Landis method as described by Gottschalk and Mylle (1956) The pressure in the micropipette and a Hansen capacitance pressure transducer was adjus ed by means of a mercury levelling bulb All connections were stiff water filled tubings Proximal as well as distal intratubular pressures were measured in several convolutions during the urine collection periods. Colloid osmotic pressure (COP) of plasma was measured directly in 25–50 μl plasma aliquots using an electronic osmometer (type HQ-66 S & E) as described by Hansen (1961)

Plasma renin was measured in 5 μl aliquots by the method of Poulsen and Jorgensen (1974) which is based on angiotensin I trapping by antibody The results are expressed as renin concentration defined as the enzymatic activity of renin uninfluenced by the individual variations in renin-substrate concentrations

Inulin and PAH clearance was calculated from the urine/plasma concentration ratios (U P_{IN}) and the volume of urine collected per minute (V_U) divided by the kidney weight (KW) according to the conventional expression

$$C_{IN} = (U\ P_{IN})\ (V_U/KW)\ (\text{ml} \times \text{min}^{-1} \times \text{g KW}^{-1})$$

End proximal tubular fluid plasma inulin ratio (F/P_{IN}) as calculated from the measured OT and proximal transit time (TT_{prox}) according to the equation (Bojesen and Leyssac 1969)

$$\ln F/P_{IN} = TT_{pro}\ OT$$

Measured OT values were all corrected for 12 reflux from pars recta Absolute rate of proximal reabsorption was calculated from the C_{IN} and F P_{IN} The rate of reabsorption in nephron segments distal to the proximal convolutions (lower nephron segments) was calculated as the difference between overall tubular reabsorption rate and reabsorption rate by the proximal convolutions.

Renal plasma flow (RPF) was calculated from the PAH-clearance (C_{PAH}) and PAH extraction (E_{PAH}) as

$$RPF = \frac{C_{PAH}}{E_{PAH}}$$

Filtration fraction (FF) was calculated as

$$FF = \frac{RPF}{C_{IN}}$$

A comparison of the absolute values of the various parameters measured in the control period between 2 groups was made by analysing the significance of difference between the mean values.

Within each group mean percentage changes were calculated as the means of the percentage changes in individual experiments

The significance of these mean percentage changes within a group as well as the significance of difference in mean changes of a parameter between two groups was calculated.

Significance of difference was analysed by student t test.

Results

Table I and II summarize the mean values of the various parameters investigated in Amytal and Inactin groups respectively The tables include both values for the control period and the means of changes observed in the steady state following a 2 5 ml saline load i v

Control systemic arterial pressure was 12 mm Hg higher in rats anesthetized with Inactin than in those given Amytal ($p < 0\ 05$) In neither group did pressure change after saline loading

Although control urine flow was significantly lower in the Inactin group than in the Amytal group ($p < 0\ 001$) the relative increase after saline was about the same (85 % and 92% respectively) Also total solute excretion rate was lower with Inactin than with Amytal but the difference was not statistically significant ($p > 0\ 05$) After saline infusion solute excretion increased significantly in both groups The individual data are given in Fig 1 and 2

Inulin clearance averaged 1 28 ml × min^{-1} × g KW^{-1} in the control period of rats anesthetized with Amytal and increased about 25 % after 2 5 ml saline (Table I) This increase was highly significant ($p < 0\ 001$) In rats given Inactin control C_{IN} was significantly lower

TABLE I Group I Data collected before and 30 min after an infusion of 2 5 ml isotonic sal ne in 250 g rats anesthetized with Amytal 15 mg/100 g b wt

	Control per od	N	Exp ch nge Abs		p
BP (mm Hg)	103 ±4	24	−3 ±3	—	N S
U ine flow (µl × min ¹ × g Kw⁻)	5 94 ±0 77	28	+5 31 ±1 19	+9 3±14 7	<0 001
Osm excr (µosm min⁻¹ g Kw⁻)	6 83 ±0 99	19	+3 69 ±0 58	+66 8±16 9	<0 001
C_{IN} (ml × m n⁻¹ g Kw⁻)	1 8 ±0 06	28	+0 5 ±0 05	+23 8± 5 1	<0 001
C_{PAH} (ml min⁻¹ g Kw⁻)	3 95 ±0 28	13	+0 93 ±0 36	+30 7±12.6	<0 05
E_{PAH} ( )	84 3 ±1 4	1	+1 81 ±1 94	—	N S
FF	0 95±0 016	12	−0 013±0 0 0	—	N S
F/P_{IN}-prox	53 ±0 08	8	+0 13 ±0 08	—	N S
Abs p ox reabs (ml min ¹ × g Kw⁻)	0 74 ±0 04	8	+0 18 ±0 0	+ 69± 4 4	<0 001
Abs reabs lower neph segm (ml m n g Kw⁻)	0 535±0 0?9	27	+0 07 ±0 040	+ 1 7± 8 5	N S
COP (mm Hg)	17 18 ±0 65	13	−1 53 ±0 25	−10 4± 1 4	<0 001
Plasma ren n (10⁴ GU ml)	3 78 ±0 65	8	−0 88 ±0 34	—	<0 05
Prox press (mm Hg)	1 80 ±0	14	+0 80 ±0 3	—	<0 0 5
Dist. press (mm Hg)	6 13 ±0 5	15	+0 6 ±0 4	—	N S

Data are g n as means ±S E.
N = number of an mals.

TABLE II Group II Data collected before and 30 min after an infusion of 2.5 ml isotonic saline in 30 g rats anesthetized with Inactin 1. 5–15 mg/100 g b wt

	Control period	N	Exp change		
			Abs		p
BP (mm Hg)	115 ±2	13	0 ±1 8		N S.
Urine flow (μl × min^{-1} × g Kw^{-1})	1 97 ±0 23	13	+1.31 ±0 ?9	+ 85 1±?0	<0 001
Osm. excr (μosm min^{-1} × g Kw^{-1})	3 87 ±0 32	9	+3 70 ±0 61	+109 9±25 9	<0 001
C_{IN} (ml × min^{-1} × g Kw^{-1})	0 92 ±0 07	13	+0 12 ±0 07	+ 16 4± 8 0	N S
F/P_{IN}-prox	2 23 ±0 08	13	−0 12 ±0 05	—	<0 05
Abs prox reabs (ml × min^{-1} × g Kw^{-1})	0 50 ±0 04	13	+0 03 ±0 05	+ 8 1± 8 7	N S.
Abs reabs lower neph. segm (ml min^{-1} g Kw^{-1})	0 413±0 033	13	+0 085±0 036	+ 0 7± 8 1	<0 05
COP (mm Hg)	16 12 ±0 29	9	−1 70 ±0 32	− 10 5± 1 9	<0 001
Plasma renin (10 GU × ml^{-1})	5 13 ±0 75	12	−1 07 ±0 69	—	N.S.

Data are given as means ±S E.
N = number of animals

(0 92 ml min^{-1} g KW p < 0 001) and in contrast to group I it did not increase significantly after saline loading (Table II) The data from the individual experiments are given in Fig. 3

End proximal tubular fluid/plasma inulin ratio (F/P_{IN}) as a measure of proximal fractional reabsorption was 2 53 in the Amytal group and tended to increase after saline but the change was statistically insignificant Proximal fractional reabsorption was significantly lower in the Inactin group (F/P_{IN} 2 23 $p < 0 025$) and it decreased significantly but moderately after saline (p 0 05 Table II)

With Amytal absolute proximal reabsorption rate increased in parallel with C_{IN} (about 27 %) the increase being highly significant (p 0 001) In contrast proximal reabsorption rate in the control period with Inactin was significantly lower (p 0 001) and it did not change after saline (Table II) This difference in response to saline between the two groups was significant (p 0 05) Data from individual experiments are presented in Fig 4 There was no correlation between the dose of Inactin and either absolute reabsorption rate or the change following saline

Reabsorption rate in segments distal to the proximal convolution (lower nephron segments) increased after saline in both groups although in the Amytal group the increase was not statistically significant Individual data are given in Fig 5

Saline infusion caused a decrease in arterial plasma COP of about 10 from a control value of about 17 mm Hg. There was no significant difference between the two groups

Plasma renin concentration decreased moderately but significantly (p 0 05) after saline in rats anesthetized with Amytal (Table I) Using paired values for renin concentration and proximal reabsorption rate between control and experimental (2.5 ml saline) periods with Amytal a highly significant inverse relationship was apparent between log renin concentra

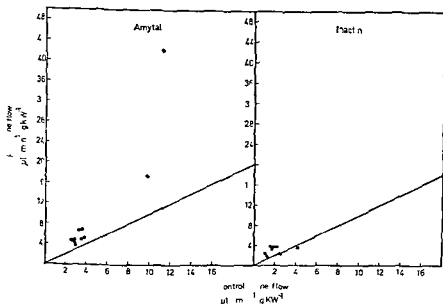


Fig. 1 Change in urine flow after 2 5 ml saline (exp period) as compared to the control period The line indicates line of identity Individual data from rats anesthetized with Amytal (left) and Inactin (right) respectively

tion and reabsorption rate (Fig 6) In the Inactin group control renin concentrations were slightly higher but the difference between the groups was insignificant In contrast to the Amytal group the decrease observed after saline was not statistically significant Nor could a relationship between renin concentration and proximal reabsorption rate be detected in the Inactin group (Fig 6)

Intratubular pressures were measured systematically only in the Amytal group (Table I)

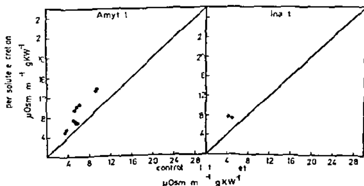


Fig. 2. Chang in solute excret on rate after 5 ml saline (e p period) as compared to the control period. The line ind cates line of identity Indi dual data from rats anesthetized w th Amytal (left) and Ina (right) respecti ely

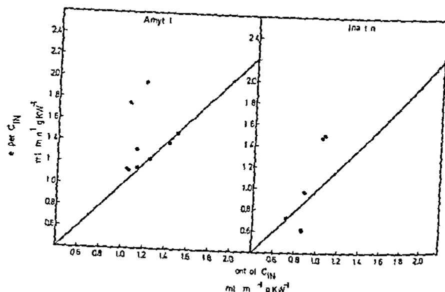


Fig 3 Change in C_{IN} after 2 5 ml saline (exp period) as compared to the control period The line indicates line of identity Individual data from rats anesthetized with Amytal (left) and Inactin (right) respectively

In the steady state following a 2 5 ml saline load both proximal and distal intratubular pressures were little altered from the control A small but significant increase averaging 0 8 mm Hg ($p < 0.05$) was observed in the proximal convolution while the mean increase of 0 6 mm Hg measured in the distal tubule was insignificant It has however been shown that prior to the new steady state the same volume expansion causes an increase of 5 6 mm Hg in proximal intraluminal pressure (Leyssac 1969)

PAH clearance increased significantly and virtually in parallel with C_{IN} (about 30

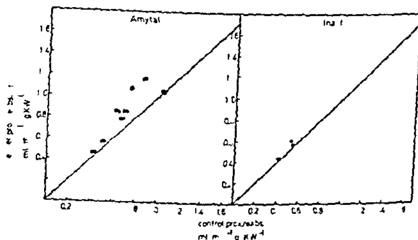


Fig. 4 Change in absolute proximal reabsorption rate after 2 5 ml saline (exp period) as compared to the control period The line indicates line of identity Individual data from rats anesthetized with Amytal (left) and Inactin (right) respectively

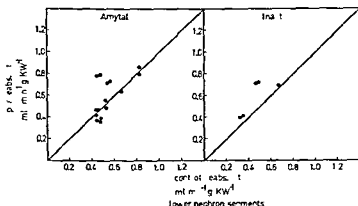


Fig. 5 Change in reabsorption rate in lower nephron segments after 2.5 saline (exp period) as compared to the control period The line indicates line of identity Individual data from rats anesthetized with Amytal (left) and Inactin (right) respectively

p<0 05) after saline given in the Amytal group (Table I) PAH extraction (E_{PAH}) remained unchanged and filtration fraction (FF) tended to fall the decrease was however not statistically significant

Discussion

The present investigation deals with two topics The first is whether or not the use of the thiobarbiturate Inactin as an anesthetic for rats has influenced the results obtained in studies on regulation of proximal tubular function The second is whether or not the oncotic pressure of the peritubular environment is a major regulating factor for proximal tubular reabsorptive capacity under physiological conditions

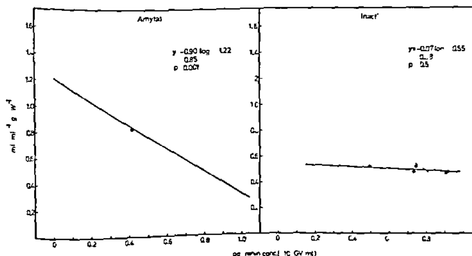


Fig. 6 Relationship between log plasma renin concentration and absolute proximal reabsorption rate in rats anesthetized with Amytal (left) and Inactin (right) Data from both control periods and after 2.5 ml saline are included The regression lines are indicated by continuous lines. The dotted line in the diagram to the right represents the regression line obtained in the Amytal group (left)

The data demonstrate that rats anesthetized with Inactin differ with respect to renal function and respond differently to a moderate saline load than those anesthetized with Amytal

In a previous study (Elmer *et al* 1972) using large numbers of rats it was concluded that compared with Amytal anesthetic doses of Inactin in the range 12–25 mg/100 g b wt inhibit proximal reabsorptive capacity This conclusion was based on the following findings Firstly a significant inverse relationship between serum Inactin concentration and proximal reabsorption rate was demonstrated secondly absolute as well as fractional proximal reabsorption was significantly lower in rats given Inactin and finally reabsorption rates were unrelated to serum Amytal concentration The conclusion was supported by *in vitro* studies indicating that Inactin concentrations similar to those measured in serum of anesthetized rats caused irreversible inhibition of isosmotic net fluid transport by rabbit gallbladder while Amytal in such concentrations did not inhibit transport (Christensen *et al* 1973) However Dev *et al* (1973) were unable to detect with micropuncture methodology any difference in single nephron filtration rate (sngfr) or proximal tubular absolute or fractional reabsorption between rats anesthetized with Inactin and Amytal nor could they find any correlation between proximal reabsorption rate and the dose of Inactin within the range sufficient for anaesthesia in their rats (10–15 mg/100 g b wt) They concluded that there was no impairment of tubular function with Inactin and further that there were no differences between the two barbiturates Unfortunately their results are not directly comparable with our previous results (Elmer *et al* 1972) because we had to use a larger range of Inactin doses for adequate anesthesia (12–25 mg/100 g b wt) possibly because our rats were older and larger (250 g b wt as compared to 160–220 g b wt) In addition their conclusions are based on many measurements made on few animals in each group serum barbiturate concentrations were not measured and considerably larger supplementary doses of Amytal were given i v rather than i p

In the present study in order to make a more valid comparison we excluded all rats which could not sleep adequately with a total Inactin dose of at most 15 mg/100 g b wt The results show in agreement with the Munich group that within this narrow range of Inactin dose (12.5–15 mg/100 g b wt) as used by the majority of investigators proximal fractional reabsorption is slightly but significantly higher than we found previously using the larger range of Inactin doses (*cf* Elmer *et al* 1972) and furthermore that absolute rates of proximal reabsorption do lie predominantly within the range of values measured in rats anesthetized with Amytal (*cf* Fig 4) Nor was there any detectable correlation between the dose of Inactin and the rate of reabsorption However when the data are compared with those obtained in rats anesthetized with Amytal a depressant effect of Inactin on tubular function becomes apparent. In the Inactin group the range of proximal reabsorption rates was relatively narrow as compared to the Amytal group all values being in the lower half of the range of reabsorption rates obtained with Amytal Furthermore after a small saline load absolute proximal reabsorption rate (and GFR) failed to increase in rats anesthetized with Inactin in contrast to a highly significant increase in these parameters observed in rats anesthetized with Amytal Thus even though proximal reabsorption rates similar to those in the lower range of values seen in Amytal rats can be obtained with low Inactin doses the

anesthetic blocks or severely reduces the proximal tubular response to the saline loading In contrast no inhibitory effect of Inactin could be detected in more distal nephron segments nor was the response to saline blunted in these segments Also the diuretic and natriuretic response to saline was similar (about a doubling) in rats given oxybarbiturate and thiobarbiturate respectively Thus the present results are in agreement with out previous observations (Elmer *et al* 1972) and are quite consistent with those reported by Dev *et al* (1973) The conclusion can be reached that the extensive use of Inactin for experimental research on regulation of proximal tubular transport is unfortunate since proximal reabsorption rate is depressed and its response to physiological stimuli is blocked by pharmacological effects of the thiobarbiturate Inactin whereas the oxybarbiturate apparently does not exert such effects

In a previous report (Elmer *et al* 1972) an inverse relationship between log renin activity in arterial plasma and spontaneous levels of absolute proximal reabsorption rate was demonstrated in Amytal anesthetized rats Such a relationship was absent in rats anesthetized with Inactin In the present study induction of an increase in tubular reabsorption by moderate saline loadings raised this inverse correlation to an even more significant level than that obtained for spontaneous variations only Such a finding is consistent with the hypothesis that the renin angiotensin system is directly involved in regulation of proximal reabsorptive capacity and thereby of GFR, under these conditions (Leyssac 1964 1965) Recently more direct evidence indicating an inhibitory effect of angiotensin II on proximal tubular reabsorption has been reported by Steven (1974 b) from peritubular capillary microperfusion experiments in Amytal anesthetized rats a depression of reabsorptive capacity by angiotensin was accompanied by an increase in proximal intraluminal pressure The opposite effect on the latter was obtained by microperfusing an angiotensin inhibitor (the competing analogue 1 Sar 8 Ala Angiotensin II) suggesting a physiological effect of endogenous angiotensin (Steven, personal communication) Finally Wiesenbaugh and Hill (1970) have shown that dogs immunized with hog renin have a diminished renal hemodynamic response to isotonic (and hypotonic) saline while excreting an increased fraction of the salt and water load as compared with normal control dogs These data also provide evidence that the renin angiotensin system is significantly involved in the renal response to saline loading

Current views strongly favour an important role of the peritubular physical factors (so-called Starling factors) for the adjustment of net proximal reabsorption rate This hypothesis would predict a depression of proximal reabsorption rate after i v infusion of saline because of the accompanying dilution of peritubular plasma proteins (oncotic pressure) unless a concomitant considerable rise in filtration fraction occurred However no evidence has been provided that filtration fraction might increase following saline on the contrary it would appear if anything to decrease This is true for whole kidney as well as superficial nephron function (Stein *et al* 1972 Bruns *et al* 1974 Barret *et al* 1973) The present study demonstrates that in the Amytal anesthetized rat in which tubular and overall renal function is found to respond adequately without detectable drug induced limitation, an i v load of 2 5 ml isotonic saline induced a 25% increment in GFR, overall tubular reabsorption and superficial proximal reabsorption rate while arterial oncotic pressure was reduced and whole kidney filtration fraction remained unchanged or fell slightly Pressure measure

in surface proximal tubules and adjacent peritubular capillaries have failed to yield evidence of any selective change in capillary hydrostatic pressure after isotonic volume expansion (Daugharty *et al* 1972) The present results are therefore incompatible with the concept that peritubular physical factors and in particular oncotic pressure play any significant role in adjustment of proximal tubular function after a moderate saline load The contrary conclusion has been reached in a number of studies in Inactin anesthetized rats (see introduction). However the fact that Cortney *et al* (1965) were unable to find inhibition of proximal tubular reabsorption even after extreme volume expansion in pentobarbital anesthetized rats and also that Steven (1974 b) in similarly anesthetized rats found no change in proximal reabsorption rate during peritubular capillary microperfusion with saline (diluting the peritubular plasma) suggests that the proximal tubular response to saline and to changes in COP depends critically upon the anesthetic used

The present results demonstrate that anesthetic doses of Inactin (12–15 mg/100 g b wt) depress proximal reabsorption rate in the hydropenic animal and attenuate the proximal tubular response to saline In contrast in Amytal anesthetized rats both proximal reabsorption and glomerular filtration rates were generally higher and responded to a modera saline load with a significant increase of about 27% The results have implications regarding the interpretation of earlier micropuncture data obtained in Inactin anesthetized rats and indicate that Inactin should be avoided for studies on regulation of renal tubular function. In addition the data are incompatible with the physical factor hypothesis of regulation of net proximal tubular reabsorption rate but are consistent with the hypothesis that the renin angiotensin system modulates proximal reabsorption rate under conditions of hydropenia and moderate saline diuresis in rats anesthetized with Amytal

The present work was supported by grants from the Danish State Medical Research Council 51 8– a 3 51 1534 *We are very grateful for the skilful technical assistance of* Anni Salomonsson and Pia S anckorg

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Acta physiol scand 1975 94 484–496

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Indomethacin Blockade of Renal PGE-Synthesis Effect on Total Renal and Tubular Function and Plasma Renin Concentration in Hydropenic Rats and on Their Response to Isotonic Saline

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Received 31 January 1975

Abstract

LEYSSAC P P P CHRISTENSEN R HILL and S L SKINNER *Indomethacin blockade of renal PGE synthesis effect on total renal and tubular function and plasma renin concentration in hydropenic rats and on their response to isotonic saline* Acta physiol scand 1975 94 484–496

The effects of indomethacin (I) a blocker of prostaglandin (PG)-synthetase was studied in rats in an attempt to elucidate the physiological role of renal PGE. Plasma I-concentrations of 13 14 μg/ml reduced renal venous plasma PGE-concentration significantly from 216 to 85 pg/ml within 45 min Urine flow and solute excretion decreased by 4 and 0 respectively while urine osmolality increased 450 mOsm. Inulin clearance (C_{In}) and proximal reabsorption rate was unaffected while renal plasma flow (RPF) decreased by 18 Plasma renin concentration decreased slightly but significantly An i v saline load equal to I b wt given to I treated rats failed to elevate significantly either urine flow solute excretion C_{In} RPF or proximal reabsorption rate but plasma renin decreased further The normal inverse relationship between plasma renin and proximal reabsorption rate was absent. The data are consistent with the concept that intrarenal PGE plays a role in adjustment of renal vascular resistance and support the concept of a physiological role of intrarenal PGE in regulating salt and water excretion The data do not support an major physiological role of PGE in regulating proximal tubular function

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Increasing evidence suggests that renal prostaglandin E_2 (PGE_2) synthesized and liberated by the kidney functions as a local hormone participating in the regulation of renal blood flow and its intrarenal distribution (for review see McGiff and Itskovitz 1973) The finding of a marked reduction in overall renal blood flow following blockade of PG biosynthesis with indomethacin (Lonigro *et al* 1973) has two important implications firstly it suggests that renal vascular resistance depends upon the action of local endogenous PGE secondly the marked effect of indomethacin cannot be accounted for in terms of changes in medullary

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blood flow alone and therefore suggests a role for cortical PGE production This latter suggestion is supported by the presence in renal lymph of PGE like material which increases in amounts after *infusion of noradenaline and angiotensin II (Fujimoto and Lockett 1970)* and by the demonstration that not only medullary tissue but also renal cortical tissue is capable of synthesizing appreciable amounts of PGE_2 (Larsson and Änggård 1973) However PGE carried to or liberated locally in the cortex will be rapidly inactivated and metabolized by its high 15 hydroxy prostaglandindehydrogenase activity (Larsson and Anggård 1973)

Besides its vascular and smooth muscle actions prostaglandins have been shown to influence transepithelial ion and water transport The effect differs between high resistance and low resistance epithelia Thus PGE enhances sodium transport but inhibits vasopressin induced water transfer across high resistance epithelia like frog skin toad bladder and mammalian collecting duct as demonstrated first by Orloff *et al* (1965) while it inhibits net salt and water transport across low resistance epithelia such as intestinal epithelium (AlAwquati and Greenough 1972 Matuchansky and Bernier 1973) and gall bladder epithelium (Leyssac *et al* 1974 a Morton *et al* 1974) A moderate inhibitory effect of PGE_1 (but not of PGE_2) on renal proximal tubular reabsorption was recently reported by Strandhoy *et al* (1974)

These findings raise the question whether or not endogenous PGE production locally in the renal cortex might be a major determinant of proximal tubular reabsorptive capacity under physiological conditions The present investigation indicates that blockade of renal PGE biosynthesis with indomethacin in anesthetized hydropenic rats causes a reduction in urine flow and solute excretion rate without detectable effect on either glomerular filtration rate (GFR) or proximal reabsorption rate within 30 min Furthermore indomethacin abolished the vascular tubular and diuretic response to a moderate isotonic saline infusion without affecting the decrease in renin release

Methods

Male SPF Sprague Dawley rats weighing 240–290 g allowed free access to food and water prior to the experiment were anesthetized with intraperitoneal (i p) injections of sodium Amytal 15 mg/100 g b wt. Anesthesia was supplemented when necessary with additional doses of 1–2 mg/100 g b wt. The rats were placed on a servo controlled heated micropuncture table maintaining body temperature about 37 C and prepared for clearance proximal tubular occlusion time (OT) and transit time (TT) measurements as described previously (Leyssac *et al* 1975)

Group I Hydropenic control rats

A control period including blood sampling serial urine collections OT and TT measurements was obtained as described in the preceding paper (Leyssac *et al* 1975) After the first control clearance period 0 75 ml of 0 9 NaCl was given i followed by a continuous infusion of 15 μl min. 30 min later the second clearance period was started including an initial blood sample urine collections OT and TT measurements and final blood samples from the renal vein and of the carotid artery

Group II Indomethacin [illegible] n to hydropenic rat

The procedure was identical to that of group I *except that 1.5 mg/kg b wt of indomethacin was included* in the 0 75 ml given i after the first control clearance period This priming dose was followed by a continuous infusion of 15 μg indomethacin/kg b wt. per min in a volume of 15 μl min (including PAH a polyfructosan) throughout the rest of the experiment.

Group III 2.5 ml isotonic saline infusion to indomethacin treated hydropenic rats

Indomethacin (1 5 mg/kg b wt) was given i v in a volume of 0 75 ml at the time of PAH and polyfructosan priming and was followed by a continuous infusion of 15 μg indomethacin/kg b wt pr min in a volume of 15 μl/min throughout the experiment The procedure thereafter followed that described for group I except that after the completion of the first clearance period 2 5 ml isotonic saline were given i v at a rate of 1 0 ml/min followed by a continuous infusion of 15 μl saline/min throughout the rest of the experiment

In some of the animals of each group a 6 ml blood sample was collected from the left renal vein at the end of the experiment for prostaglandin E analysis In order to avoid *in vitro* PGE synthesis after the blood collection in animals not given indomethacin (group I) the blood collection cannula was connected to a 3 way stop-cock carrying one syringe containing 700 μl indomethacin solution (0 5 mg/ml) and a second larger syringe for blood withdrawal The indomethacin solution was gradually injected into the aspirated blood during collection assuring instant mixing and a final indomethacin concentration of about 15 μg/ml. Blood samples were immediately cooled to 0 C and centrifuged

After blood collections the left kidney was removed drained and weighed

Inulin (polyfructosan) *PAH osmolality colloid osmotic pressure* (COP) and *plasma renin* were measured as described in the preceding paper (Leyssac *et al* 1975)

Indomethacin in plasma was determined by a fluorimetric method based on that of Holt and Hawkins (1965) A 50 μl aliquot of the plasma sample was diluted with an equal volume of citrate buffer pH 5 15 and thereafter extracted by shaking with 2 2 ml of n heptane containing 5 v/v pentanol for 1 min. After centrifugation 2 ml of the heptane phase were shaken with 1 1 ml of pH 11 8 Sorensen glycine buffer centrifuged and 1 ml of the buffer phase diluted with 1 ml of the glycine buffer (total dilution 48 7)

The fluorescence of this solution was then measured in 1 cm-cuvettes at 19 5 C in an Aminco Bowman spectrophotofluorimeter with the excitation and emission monochromators set at 290 nm and 390 nm respectively

Prostaglandin E_2 in renal venous plasma was measured either with the double isotope derivative method of Bojesen and Buchhave (1972) or by a *modification* of the radioimmunoassay (RIA) method described by Caldwell *et al* (1971) Complete details of the method will be published in a later paper but the significant modifications were as follows

By immunization with PGE_2 an antibody was produced with the following cross reactivity PGF_2 100% E_1 14 1% A_2 2 8 A_1 0 7 B_2 0 3 F_2 0 06

Purification of PGE. Although the antibody was rather specific for PGE_2 a purification process was required before its use in RIA in order to be sure that only PGE_2 and E_1 were measured Individual sample recovery was determined by adding ^{3}H PGE (1 000 cpm) to each 2 ml serum aliquot The prostaglandins were then extracted from serum with acetone concentrated by evaporation acidified to pH 3 and extracted into ethyl acetate After reextraction with Na bicarbonate 0 1 M pH 8 3 acidification and reextraction with ethyl acetate chromatographic separation was performed on a micro-column of Sephadex LH 0 (10 0 5 cm) with the solvent system dichloremethan–methanol (98 2) This procedure gives a complete separation of PGE from PGA + PGB and PGF respectively

An aliquot of the PGE fraction from each serum sample was used for recovery the rest was used for RIA

RIA Since it was found that the eluate from the Sephadex column displaced the standard curve an aliquot of column-eluate without PG was added to each standard in order to correct for solvent blanks at any dose level of the standard curve The assay procedure thereafter followed the standard procedure for prostaglandin radioimmunoassay

Calculations and statistical analyses were performed as described in the preceding paper (Leyssac *et al* 1975)

Results

The dose of indomethacin which would give and maintain a plasma concentration of 10 15 μg/ml was determined in a preliminary series of experiments. It was found that 1.5 mg of indomethacin per kg b wt followed by a continuous infusion of 15 μg per kg b wt and min gave a plasma concentration of 13 6 2 5 (S D) μg/ml which was maintained constant for more than 2 h

PGE$_2$-concentration in renal venous plasma of 3 control rats (group I) measured by the

TABLE II. Group II. Data collected before and 30 min after infusion of indomethacin in 250 g rats.

	Control period	N	Change after indomethacin		
			Abs	%	p
BP (mm Hg)	109 ± 2	75	−7 ±2	—	<0.001
Urine flow ($\mu l \times min^{-1} \times g\ Kw^{-1}$)	6.07 ±0.63	20	−3.00 ±0.53	−41.7±3.4	<0.001
Osm. excr ($\mu osm \times min^{-1} \times g\ Kw^{-1}$)	8.55 ±0.80	14	−1.73 ±0.19	−20.3±5.7	<0.001
U_{osm} ($mosm \times kg^{-1}$)	1.803±125	14	+450 ±1.1	—	<0.005
C_{IN} ($ml \times min^{-1} \times g\ Kw^{-1}$)	1.34 ±0.05	16	+0.0. ±0.07	+ 1.7±5.1	N.S.
C_{PAH} ($ml \times min^{-1}$ g Kw^{-1})	4.14 ±0.23	7	−0.74 ±0.14	−17.7±.8	<0.001
E_{PAH} ( )	78.3 ±3.3	7	+4.9 ±2.7	—	N.S.
FF	0.275±0.031	7	+0.054±0.0.5	—	N.S.
F/P_{IN}-prox.	2.51 ±0.09	20	+0.17 ±0.11	—	N.S.
Abs. prox. reabs (ml min^{-1} × g Kw^{-1})	0.789±0.039	16	+0.051±0.050	+ 7.5±6.5	N.S.
Abs. reabs lower neph. segm (ml min^{-1} × g Kw^{-1})	0.539±0.0.8	16	−0.001±0.037	− 0.5±6.0	N.S.
Plasma renin (× 10^{-4} GU × ml^{-1})	1.88 ±0.25	14	−0.57 ±0.21	—	<0.05

Data given as means ± S.E.
N = number of animals

The decrease in urine flow after indomethacin was accompanied by a significant increase in urine osmolality (mean 450 mosm). Individual data for controls and treated rats are presented in Fig. 3.

Neither inulin clearance, F/P_{IN}, absolute proximal reabsorption rate, nor rate of reabsorption in tubular segments distal to the proximal convolution changed significantly after indomethacin. Individual data on proximal reabsorption rate obtained in the control group for comparison with those obtained in rats given indomethacin are presented in Fig. 4.

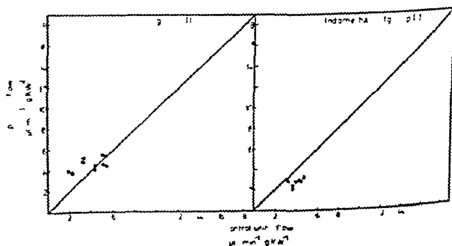


Fig. 1. Change in urine flow between period I and period II in control rats (left) and in rats given indomethacin after period I (right). The line represents the line of identity.

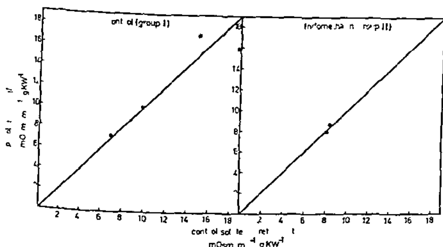


Fig 2 Change in solute excretion rate between period I and period II in control rats (left) and in rats given indomethacin after period I (right) The line represents line of identity

Plasma renin concentration decreased after indomethacin in 13 of 14 expts The mean change was small but statistically significant In control rats (Table I) plasma renin tended to increase but the change was statistically insignificant The difference in response between the two groups was highly significant ($p < 0.005$)

The effect of indomethacin on renal plasma flow and filtration fraction was investigated in a small additional series of group-II experiments RPF as estimated from C_{PAH} and E_{PAH} decreased most significantly after indomethacin E_{PAH} did not change but C_{PAH} decreased by 17 7 % ($p < 0.001$) (Table II) FF was 0 275 and increased in 6 of 7 experiments to on an average 0 329 a value equal to that obtained in rats treated with indomethacin from the start of the experiment (*cf* Table III) However the increase was quite variable and with the small number of experiments it was not statistically significant

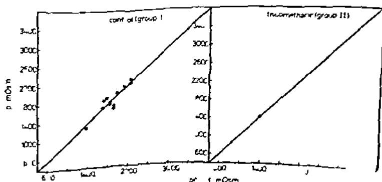


Fig 3 Change in urine osmolality between period I and period II in control ra ... indomethacin after period I (right) The line indicates line of identity

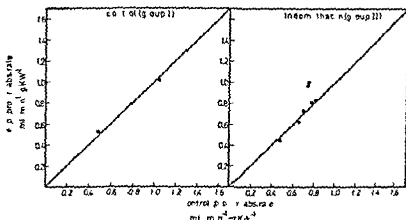


Fig. 4 Change in absolute proximal reabsorption rate between period I and period II in control rats (left) and in rats given indomethacin after period I The line indicates line of identity

Group III Renal response to saline infusion in indomethacin treated rats

The effect of expansion of the extracellular space with 2 5 ml saline (corresponding to about 1% of b wt) in indomethacin treated rats (Table III) may be compared with the effect observed in a parallel series of control rats anesthetized with Amytal (Leyssac *et al* 1975) in which the experimental procedure and conditions were identical

Systemic arterial pressure which increased moderately during the saline infusion had returned to its control value in the experimental steady state period

TABLE III Group III Data collected before and 30 min after infusion of _ 5 ml isotonic saline in _50 g rats treated with indomethacin.

	Control period	N	Change after saline		
			Abs	•	p
BP (mm Hg)	116 ±	21	−3 ± 1	—	N.S.
Urine flow (μl min^{-1} g Kw^{-1})	5 41 ±0 51	0	+0 9 _ 0 16	71 _ 15 6	N.S.
Solute excr rate (μosm min^{-1} g Kw^{-1})	8 79 ±0 76	8	+ 4 ± 1 36	31 5 16 5	N.S.
C_{IN} (ml min^{-1} g Kw^{-1})	1 45 +0 06	1	+0 03 0 08	5 6 5 8	N.S.
C_{PAH} (ml min g Kw^{-1})	3 79 ±0 6	8	+0 43 ± 0	+11 9 5 5	N.S.
E_{PAH} ( )	83 6 ± 7	7	+ 9 _ 1	—	N.S.
FF	0 3 3±0 017	7	+0 006 0 017		N.S.
F/P_{IN} prox.	4 ±0 09	21	0 1 ±0 07		<0 05
Abs. prox. reabs. (ml min^{-1} g Kw^{-1})	0 833 0 041	1	0 0 8 +0 051	−1 63 6 9	N S
Abs. reabs lower neph. segm. (ml min^{-1} g Kw^{-1})	0 618 _0 035	21	+0 064 0 044	+15 4± 7 0	N S N.S.
U_{osm} (mosm kg^{-1})	175 − 114	1	10 _ 68	111 _ 0	<0 001
COP (mm Hg)	16 9 0 39	16	−1 80 ±0 35		
Plasma renin (10^{-4} GU × ml^{-1})	90 0 40	18	−0 57 0 3	—	<0 05

Data given as means ±S E.
N = number of animals.

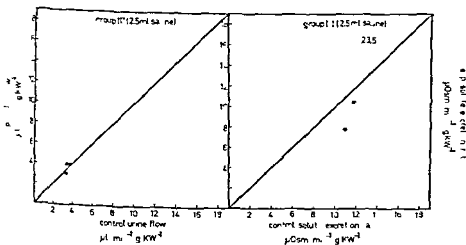


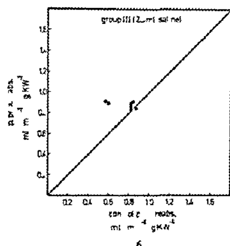
Fig. 5 Data from rats pretreated with indomethacin. Change in urine flow (left) and solute excretion rats (right) after 2.5 ml saline (exp period) as compared to the control period

Urine flow and solute excretion rate increased inconsistently after saline infusion (mean +79 and +31% respectively) These increases were statistically insignificant and should be compared with the 92% and 67% increases previously reported for control rats (Leyssac *et al* 1975) The difference in urinary flow response between the present indomethacin-treated rats and previous control rats was significant ($p < 0.01$) but the change in solute excretion rate was not significantly different in the present series as compared with previous controls Individual data from the present group III are given in Fig 5

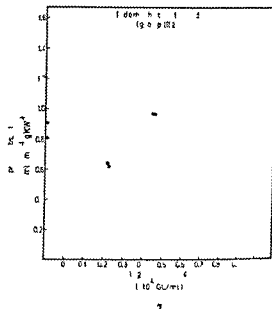
Inulin clearance as well as absolute proximal reabsorption rate was slightly higher in the control period of indomethacin treated rats than in both the present control group (Table I) and the previously reported control group (*cf* Leyssac *et al* 1975) but the difference was not statistically significant However whereas C_{IN} and absolute proximal reabsorption rate increased significantly in previous control rats with 2.5 ml saline infusion both parameters failed to increase in indomethacin treated rats The differences in response between the two groups were significant for C_{IN} $p < 0.025$ for proximal reabsorption rate $p < 0.001$ Individual data on proximal reabsorption rates in group III experiments are presented in Fig. 6

Proximal fractional reabsorption as estimated from the end proximal F/P_{IN} ratio in the control period was not significantly different from that measured in control rats (period I group I) it decreased slightly but significantly following saline infusion in the present group III Absolute reabsorption rate in lower nephron segments tended to increase after saline but the change was insignificant as it was in previous control rats

Renal plasma flow (RPF) as estimated from C_{PAH} and E_{PAH} also failed to increase significantly in response to 2.5 ml of saline in group III rats given indomethacin E_{PAH} was the same (84 %) as in previous control rats and did not change Filtration fraction was 0.3_3 a value slightly higher than but not significantly different from that in previous control rats (0.295 ± 0.016) and it did not change after saline infusion



6



7

Fig 6 Data from rats pretreated with indomethacin Change in absolute proximal reabsorption rate after 2 5 ml saline (exp period) as compared to the control period

Fig 7 Data from rats pretreated with indomethacin Log plasma renin concentration versus absolute proximal reabsorption rate Data both from control periods and after 5 ml saline are included

Plasma renin concentration (2 90 ± 0 40 10⁻⁴ GU/ml) was not significantly different from that measured in control rats it decreased slightly after 2 5 ml saline the decrease being significant at the 5 % level However any relationship between log plasma renin concentration and proximal reabsorption rate like that previously demonstrated for control rats (Leyssac *et al* 1975) was absent in rats treated with indomethacin as apparent from Fig. 7 Plasma oncotic pressure averaged 16 8 mm Hg a value not significantly different from that previously reported for control rats also the decrease of about 10 % following 2 5 ml saline was equal to that obtained in control animals

Discussion

In hydropenic control rats (group I) neither inulin clearance proximal fractional or absolute reabsorption nor urine flow and solute excretion rate changed significantly between the first and second clearance period 30 minutes apart if anything the proximal reabsorption rate increased This finding is at variance with the progressive decrease in proximal reabsorption rate after induction of anesthesia with Amytal reported by Dev *et al* (1973) The reason for this discrepancy is probably the fact that the latter group of investigators gave higher supplementary doses of Amytal by an intravenous route during the course of their experiments than those given intraperitoneally in our laboratory

Indomethacin an inhibitor of prostaglandin synthetase has been widely used as a tool to assess the physiological role of prostaglandins in various organs However the inter

pretation of such studies is difficult because indomethacin has been shown to have eff other than PG synthetase blockade Flores and Sharp (1972) deomnstrated a ..0 inhibition of cyclic nucleotide phosphodiesterase by concentrations of indometha in similar to those obtained in the present study Similarly Albert and Handler (1974) ha e st that indomethacin (and polyphloretin phosphate) not only enhances the toad bladder response to vasopressin but also to cyclic 3 -5 adenosine monophosphate (cAMP) In the presence of exogenous PGE_1 indomethacin had no effect on the response to vasopressin still increased the response to cAMP

In the present study indomethacin blocked PGE_2-biosynthesis and release to the venous blood. This effect was accompanied by a moderate fall in renal plasma flow (RPF while urine flow and solute excretion rate was markedly reduced without any detectable change in the rate of glomerular filtration (C_{IN}) This finding agrees with previous observations by Aiken and Vane (1973) Under the present antidiuretic conditions collecting duct osmolality is in near complete equilibrium with the papillary intersitium (e g Gottschalk and Mylle 1959) The marked reduction in urine flow and solute excretion in concert with elevation of urine osmolality by on an average 450 mOsm therefore suggests increased concentrating capacity rather than urine flow reduction due solely to reduced filtration pressure Furthermore this vasopressin like effect of indomethacin which is directly opposite to the renal effects of exogenous PGE_2 (Johnston *et al* 1967 Vander 1968) is entirely consistent with the well-documented hypothesis that PGE inhibits vasopressin induced effects on high resistance epithelia such as toad bladder and collecting duct epithelia by interference with membrane adenyl cyclase (Orloff *et al* 1965 Grantham and Orloff 1968 Ramwell and Shaw 1970 Ozer and Sharp 1972) but the present results do not permit any distinction between the contributions to this vasopressin like effect of potentiation of vasopressin on the collecting duct epithelium on the one hand and reduction in medullary blood flow on the other (Itskovitz *et al* 1974) Nor can it be excluded that the effect results partly from blockade of cAMP breakdown due to phosphodiesterase inhibition by indomethacin such an effect may have exaggerated the present response to blockade of PGE synthesis However the results are consistent with the suggestion that endogenous PGE may play a significant role in regulation of solute and water excretion in hydropenic rats

The attenuation of the haemodynamic as well as diuretic response to a moderate isotonic expansion of the extracellular space observed in the present indomethacintreated rats (group III) suggests that PGE is physiologically involved in the renal tubular and/or vascular response to moderate volume expansion but again it cannot be excluded that an enhanced response to cAMP caused by indomethacin rather than a depression of PGE synthesis was responsible for this failure to respond significantly to saline Rosenthal *et al* (1974) have shown a similarly reduced capacity to excrete salt and water in response to loading in rats deprived of PGE precursors in which pharmacological effects of indomethacin was excluded. This latter observation, therefore lends support to the conclusion that the attenuation of the renal response to saline in rats given indomethacin is primarily a consequence of removal of endogenous PGE_2

The absence of any detectable change in absolute proximal reabsorption rate after effective blockade of PGE synthesis by indomethacin in hydropenic rats argues against a

role of endogenous PGE in the physiological adjustment of proximal tubular function under these conditions This does not of course exclude such a role under other especially pathophysiological conditions in which PGE release may be significantly augmented

An unexpected finding was that indomethacin treatment of hydropenic rats resulted in a moderate but significant depression of renin release concomitant with a reduction in RPF solute and water excretion indicating an effect of endogenous PGE and/or indomethacin on renin release from the juxtaglomerular cells However even in indomethacin treated rats a moderate isotonic volume expansion resulted in a further significant decrease in renin release similar in magnitude to that observed in previous control rats (Leyssac *et al* 1975) In the indomethacin treated rats the hemodynamic (RPF) response to saline was blunted suggesting that neither PGE_1 nor preglomerular vasodilatation as such is required for this change in renin release

Evidence has been previously presented supporting a role for the renin angiotensin system in the regulation of proximal tubular reabsorptive capacity (and thereby in GFR) in non diuretic and moderately saline diuretic rats (Leyssac *et al* 1975) In the present study indomethacin induced a fall in renin release without detectable increase in absolute proximal reabsorption rate furthermore the inverse relationship between log plasma renin concentration and proximal reabsorption rate observed in control rats (Elmer *et al* 1972) and rats given a moderate saline load (Leyssac *et al* 1975) was absent in indomethacin treated animals Accepting a causal relationship between the alterations in plasma renin concentration and proximal reabsorption rate in animals not treated with indomethacin the absence of such a relationship in indomethacin treated rats indicates that indomethacin interferes with the normal proximal tubular response to local angiotensin this might be either directly or through blockade of endogenous PGE_2 synthesis or other action There does appear to be some disturbance in tubular function because whereas proximal fractional reabsorption (end proximal F/P_{IN} ratio) remained unchanged or tended to increase after 2 5 ml saline in Amytal anesthetized control rats (Leyssac *et al* 1975) it decreased in indomethacin treated animals in response to an equal load (Table III) It seems worthwhile considering whether this might be due to a change in sensitivity to the local action of angiotensin but for this to operate indomethacin would have to exert a marked sensitizing effect to the inhibitory action of angiotensin even at normal intrarenal levels of the latter The inhibitory effect of indomethacin on nucleotide phosphodiesterase (Flores and Sharp 1972) with local accumulation of cAMP could be responsible for such an effect Evidence favours the suggestion that elevated levels of cAMP depress fluid transfer across isosmotically transporting epithelia Thus Fülgraff and Meiforth (1974) in a micropuncture study demonstrated an inhibition of proximal reabsorption rate by cAMP and in the intestinal epithelium net transport inhibition by PGE was augmented by theophylline (Pierce *et al* 1971) also suggesting that increased levels of cAMP inhibits isosmotic fluid transport Evidence that transport effects of angiotensin on colonic epithelium are mediated by elevating the level of cAMP has been provided by Hornych *et al* (1973) a conclusion apparently opposed by results reported by Davies *et al* (1972) However a reason for this disagreement in results could be the fact that the latter group tested effect of cAMP while Hornych *et al* used the more permeable dibutyryl-derivative (Db-cAMP) In the rabbit gall bladder PGE was also found to be a

potent inhibitor of isosmotic transport and indomethacin potentiated the effect of added PGE about one order of magnitude (Leyssac *et al* 1974 a) This potentiation by indomethacin is again consistent with a blocking effect on phosphodiesterase

Contrary to this suggestion is our previous finding in the gall bladder that indomethacin actually depressed the inhibitory response to angiotensin (Leyssac *et al* 1974 b) However the data suggested that in the gall bladder the inhibitory effect of angiotensin was mediated by liberation of endogenous PGE_2 Indomethacin blocked this liberation Even though transport characteristics are very similar to those of the gall bladder the renal proximal tubule may well differ in this respect having specific receptor sites for angiotensin on the membrane bound adenyl cyclase in contrast to gall bladder epithelium which may require other specific hormones as mediators for its transport regulation

The present work was supported by grants from the Danish State Medical Research Council 51 1935 51 2672, 512 3079 51 3636 and 51 1534 (to Professor S L. Skinner) and from Miss I A Brandts Legat, for which the authors wish to express their gratitude The skilful technical assistance of Mrs Anni Salomonson and Mrs Pia Svaneborg is also gratefully acknowledged

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Cardiovascular Responses to Changes in Carotid Sinus Transmural Pressure in Man

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Received 6 February 1975

Abstract

Bjurstedt H G Rosenhamer and G Tydén *Cardiovascular responses to changes in carotid sinus transmural pressure in man* Acta physiol scand 1975 94 497–505

To study the relative importance of cardiac and peripheral effector mechanisms in the carotid sinus baroreflex in man cardiovascular responses to equal changes of the carotid sinus transmural pressure (Ptm) in either direction of the normal were recorded and compared in eight physically well trained young male volunteers In both the supine and the 70 head up position a decrease in Ptm produced a more potent reflex response of the systemic arterial pressure than did a similar increase in Ptm Whereas the arterial pressure response to increased Ptm was due solely to a reduction in vascular resistance a significant increase in cardiac output contributed to the more potent pressor response to a decrease in Ptm and thus to the predominantly antihypotensive properties that characterize the carotid sinus baroreceptor control system in man However since combined beta adrenergic and parasympathetic blockade abolished the effect of reduced Ptm on cardiac output without greatly impairing the blood pressure response it is concluded that adjustments in cardiac output are not of critical importance in the buffering function of the carotid sinus baroreceptors Autonomic cardiac blockade exaggerated the fall in cardiac output on head up tilt, the arterial pressure remaining unaffected due to a compensatory increase in systemic vascular resistance

Strategically located near the base of the skull the carotid sinus baroreceptors act as an important anti G device in man by buffering the large arterial pressure changes that would otherwise occur at head level with changes in posture It is generally recognized that this buffer function is in part exercised by changes in total peripheral resistance mediated reflexly by variations in the tonic activity of sympathetic vasoconstrictor fibers The extent to which reflex alterations in cardiac output may contribute to such buffering of arterial pressure changes in man is uncertain In patients under general anesthesia Carlsten *et al* (1958) observed that electrical stimulation of the surgically exposed carotid sinus nerve produced bradycardia and decreased pulse pressure this may have been indicative of a reduction in cardiac output Measurements of cardiac output during similar stimulation using implanted electrodes have shown moderate decreases in this variable in unanesthetized hypertensive (Tuckman *et al* 1966) and normotensive (Epstein *et al* 1969) patients However the ef of electrical stimulation of the sinus nerves result from both chemoreceptor and b

tor fiber activity which complicates their interpretation A more physiological method of stimulating the carotid sinus baroreceptors in man was first devised by Ernsting and Parry (1957) who increased the carotid sinus transmural pressure by decreasing the pressure in an airtight box enclosing the neck These authors tested the effects of subatmospheric pressures down to −80 mm Hg, but found no significant effect on cardiac output as measured on two occasions by the direct Fick method Bevegård and Shepherd (1966) who used the same method to stimulate the carotid sinus baroreceptors found with the dye dilution technic in six subjects resting in the supine position that subatmospheric pressures in the −20 – −60 mm Hg range produced an average decrease in cardiac output of 10–12%

No information is available as to the relative effects of positive and negative changes in carotid sinus transmural pressure on the cardiac output in man Such bidirectional pressure changes can be induced by the use of a pressure/suction helmet enclosing the head as well as the neck a method described by Thron *et al* (1967) In the present study we have used this method to assess the relative contributions of cardiac output and systemic vascular resistance to arterial pressure responses following a given change in carotid sinus transmural pressure to either side of the normal Observations were made on healthy subjects in the supine and 70 head up position and to further study the role of cardiac function in the arterial pressure responses before and after autonomic blockade of the heart

Material and Method

Eight healthy physically well trained male volunteers aged 21 31 years served as subjects Functional and dimensional data are given in Table I

The carotid arterial stretch receptors were exposed to different transmural pressures according to the method of Thron *et al* (1967) using a plexiglass helmet enclosing the neck and the head (Fig 1) The thoracic aperture was sealed airtight by a rubber gasket taped to the skin The subject breathed air at normal atmospheric pressure through a mouthpiece attached to a hole in the helmet by means of a short rubber tube To achieve the desired negative and positive pressures the helmet was connected via pressure regulators to a vacuum pump and to a tank of pressurized air respectively A strain gauge manometer was used for monitoring of the pressure in the helmet The time required to reach the desired pressure could be arbitrarily chosen and was kept at about 4 s.

Intraarterial pressure at heart level cardiac output by the dye dilution method and heart rate were obtained by methods described elsewhere (Bjurstedt, Rosenhamer and Tydén 1974)

Design of experiment During the course of 1 2 weeks preceding the day of the experiment at least preliminary sessions were conducted on separate days with each subject. During these sessions the subject was exposed to [illegible] positive and negative pressures over the head and neck similar to those used in the actual [illegible] These periods served to accustom the subject to the subjective sensations involved in the procedure

The actual experiment commenced with a 15 min period of rest in the supine position followed by two min periods during [illegible] pressures of 40 mm Hg and 40 mm Hg were successively applied in the helmet, separated by [illegible] period of normal atmospheric pressure The subject was then tilted to a 70 head-up position an[illegible]d [illegible] rest for 6 min before the above-mentioned procedure was repeated

Six of the subjects par[illegible] a second experimental session on a different day using the same experimental protocol except [illegible] combined parasympathetic and beta adrenergic blockade was induced initially by the [illegible] administration of 0.03 mg kg of atropine and 0 5 mg/kg of propranolol

Two consecutive indicator-dilution curves were recorded in each of the min positive and neg[illegible] pressure periods one after 30 [illegible] and one after 1 min 30 s Prior to these periods two control determinations of cardiac output were made with normal atmospheric pressure in the helmet in the supine as well as in the head-up position Heart rate and arterial pressure were recorded continuously throughout the experiments.



Fig 1 Plexiglass helmet for application of positive and negative pressures over the neck (subject breathing room air at normal atmospheric pressure through tube connected to frontal aperture)

Calculations Cardiac output was estimated from the mean values of the curves recorded in each experimental condition Heart rate and arterial mean pressure were averaged during the inscription of each indicator dilution curve and in each experimental condition the mean of the averaged values was used for the calculations Total peripheral resistance was calculated as mean arterial pressure (mm Hg) divided by cardiac output (ml/s) The statistical significance of differences between mean values were evaluated by applying the t test to the intraindividual differences (*cf* Fisher 1948)

Results

Table II shows the control values (means and ranges) of cardiovascular variables in the supine and 70 head up positions before and after combined parasympathetic and beta adrenergic blockade The circulatory adjustments noted after a 40 mm Hg change in the

TABLE I Function l and dimensional data BSA = body surface area from nomogram of Dubois and Dubois (1916) HR = heart rate after 15 min of supine rest, MAP = mean arterial pressure in mm Hg at heart level after 15 min of supine rest

	Age years	Weight kg	Height cm	BSA m²	HR		MAP	
					Supine	Head up	Supine	Head up
G T	28	73	190	1 99	64	89	10_	113
I G	3	76	184	1 98	49	66	96	100
M R	–	80	190	2 07	55	9	85	89
B G	25	74	179	1 9_	61	85	96	101
J P	4	63	173	1 75	71	83	99	114
M S	1	73	185	1 95	70	86	96	95
B H	–	63	178	1 78	58	83	95	10
L L	31	67	18	1 86	59	80	93	96

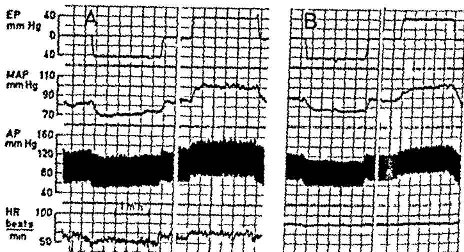


Fig 2 Segments of recordings from subject MR showing effects of changing the external pressure over the neck (EP) on mean arterial pressure (MAP) btained electronically from recorded arterial pressure in the radial artery (AP) and heart rate before (A) and after autonomic cardiac blockade (B)

helmet pressure positive as well as negative before and after autonomic blockade and in the supine as well as the 70 head up position are given in Table III

Responses to decreased external pressure on the neck

A Before autonomic blockade A typical reflex response to a 40 mm Hg decrease of the external pressure over the neck in the supine position is shown in Fig 2 A left panel Mean arterial pressure fell abruptly showing a slight overshoot and a partial recovery during the 2 min period of exposure Heart rate also decreased and remained lowered Average decrease in mean arterial pressure in the supine position was 8 mm Hg corresponding to 9 % Heart rate decreased 6 while there was no statistically significant change in cardiac output Total peripheral resistance decreased 12 %

In the 70 head up position decreased helmet pressure evoked an 8% fall in mean arterial pressure and a 9 % decrease in total peripheral resistance whereas heart rate and cardiac output showed no significant changes

B After autonomic blockade Fig 2 B (left panel) exemplifies the reflex response in the supine body position to a 40 mm Hg decrease in external pressure over the neck after combined parasympathetic and beta adrenergic blockade Mean arterial pressure fell 8 in the supine and 11 in the head up position Heart rate changes were almost abolished in both body positions As before autonomic blockade there were no significant changes in cardiac output Total peripheral resistance showed a tendency to decrease the change being statistically significant only in the head up position (Table III)

Responses to increased external pressure on the neck

Reflex responses obtained by increasing the external pressure on the neck by 40 mm Hg in the supine position before and after autonomic cardiac blockade are exemplified in Fig. 2 A and B right panels

TABLE II Control values of cardiovascular variables recorded in the supine and head up positions before and after autonomic blockade of the heart (neck exposed to normal atmospheric pressure) MAP = mean arterial pressure in mm Hg HR = heart rate in beats min CO = cardiac output in l/min TPR = total p ripheral resistance in mm Hg sec/l

		MAP	HR	CO	TPR
Without autonomic blockade	Supine	95 (85–10?)	62 (49–71)	7 6 (6 8–8 8)	(76 0 87)
	70 head up	102 (89–114)	84 (66–9)	5 6 (5 0–7 0)	7)
With autonomic blockade	Supine	99 (90–107)	88 (85–9?)	8 8 (7 0–10)	( )
	70 head up	104 (95–117)	91 (86–100)	4 3 (3 3–4 9	( )

A Before autonomic blockade In general the responses of the arterial me ure an the heart rate to 40 mm Hg increase in external pressure were stronger than they were with a corresponding decrease in external pressure They were also accompanied by significant increases in the cardiac output In the supine position mean arterial pressure and heart rate increased 17 and 16 % respectively while cardiac output increased 10% and total peripheral resistance 7 %

In the 70 head up position mean arterial pressure rose by 15% Heart rate and cardiac output increased 6 % and 10% respectively while there was no statistically significant change in total peripheral resistance (Table III)

B After autonomic blockade Following combined parasympathetic and beta-adrenergic blockade increased external pressure on the neck caused mean arterial pressure to rise 12 in the supine and 14% in the head up position with little change in heart rate Cardiac out put was not affected either in the supine or the head-up position whereas calculated total peripheral resistance increased 14 and 15 % respectively both differences being just above the $p < 0.05$ level

TABLE III Cardiovascular responses (M ± S E) to positive and negat e changes in the external pressure on the neck (for ontrol alues see Table II) $p < 0.001$ $p < 0.01$ $p < 0.05$

		MAP mm Hg	HR beats min	CO l/min	TPR mm Hg ec/ml
−40 mm Hg w thout autonomic blockade n = 8	Sup e	−8 ±1	−4 0 ±1 ?	+0 3±0 4	−0 09+0 04
	0 head up	−8 ±	−1 9 ±1	0 0±0	0 10+0 04
−40 mm Hg with autonomic blockade n = 6	Supine	8 ±1	−0 8 ±0 3	−0 1±0	−0 05 0 03
	70 head up	11±	−1.0 ±0 4	−0 1±0 1	0 11+0 04
+40 mm Hg w thout autonomic blockade n = 8	Sup	+16±	+10 1±1 4	+0 8±0	0 05 0 0
	70 head up	15±	+4 8 ±1 7	+0 6±0	0 04 0 0
+40 mm Hg with autonomic blockade n = 6	S ine	−1 ±	+1 3 ±0 4	−0 1±0 4	+0 10±0 05
	70 head up	+14±3	+0 5 ±0 5	0 0±0 2	+0. −

Discussion

When the external pressure over the head and neck is decreased the driving pressure in the enclosed vascular segments approximates the difference between inflow and outflow pressures except initially for as long as it takes for the enclosed distensible veins to fill and the pressure in these vessels to approximate the outflow pressure so that flow is resumed Thus, when steady state conditions have been reestablished a subatmospheric helmet pressure does *not* affect the driving pressure in cerebral circuits From a hemodynamic point of view this situation is however basically *different from that obtaining* with a positive pressure in the helmet In the letter case the pressure in the downstream ends of the enclosed vascular segments is close to that of the surrounding pressure rather than to the outflow pressure due to the waterfall effect which characterizes the outflow from collapsible veins when the surrounding pressure is higher than the outflow pressure (*cf* Permutt and Riley 1963) This means that in the present experiments with a positive pressure in the helmet the transmitted portion of this pressure was lost from the normal driving pressure (inflow pressure minus outflow pressure)

The possibility has been considered whether with a pressure of +40 mm Hg in the helmet the associated reduction of the normal driving pressure in cerebral circuits may have produced a systemic vascular response due to underperfusion and ischemia in medullary centers The situation is hemodynamically similar to that *obtaining* when the cerebrospinal fluid (CSF) pressure is increased the cerebral driving pressure in this case being reduced by an amount corresponding to the surrounding CSF pressure However Greenfield and Tindall (1965) found that an increase in the CSF pressure in man of 68 mm Hg (with a corresponding reduction of the driving pressure in cerebral circuits) reduced cerebral blood flow by *only* 25% and did not affect either the arterial blood pressure or the heart rate It is *unlikely* therefore that the much smaller increase in surrounding pressure produced in the present experiments by the application of a pressure of 40 mm Hg in the helmet could have provoked a cerebral ischemic *pressure response Furthermore* Sagawa Ross and Guyton (1961) observed in dogs that with the *carotid* sinus *nerves* sectioned and the cerebral arterial circulation isolated from the systemic circulation no such response occurred until the cerebral perfusion pressure fell below 40 mm Hg

Evidence has been presented by Thron *et al* (1967) that with the present technic the pressure transmitted to the wall of the carotid sinuses is linearly related to the applied helmet pressure when the latter is changed in the range 60 to 60 mm Hg *By applying* positive and negative pressure of 40 *mm Hg in the helmet equal* transmural pressure changes in opposite directions from the normal could thus be *produced* and their cardiovascular effects compared The observation that decreasing the transmural sinus pressure produces a much stronger arterial pressure response than when the transmural pressure is increased by the same amount is in agreement with the results of Thron *et al* (1967) and Stegemann, Busert and Brock (1974) The 17 rise and 9 fall in mean arterial pressure *that* resulted in supine subjects *from* changing the external pressure by *40* mm *Hg in* the positive and *negative direction respectively* are nearly identical with the results reported by these authors In the present experiments similar results were obtained in the head up position the cor

responding arterial pressure changes being +15 and −8% Our results therefore support the notion that the buffering effect of the carotid sinus baroreceptors i e their negative feedback role in response to a change in intrasinus pressure is clearly stronger following a fall than a rise in this pressure That the pressure response to a given decrease in transmural sinus pressure was not stronger in head up tilt than in the supine position may be explained by the lowering of intrasinus pressure on standing up being too small to allow an increased baroreceptor responsiveness to stand out

The observation that the carotid baroreflex acts as a predominantly antihypotensive mechanism in man refers to an intact system in which the aortic receptors tend to minimize the effects of changes in carotid baroreceptor activity One can only speculate whether the less potent blood pressure response to an increase in transmural sinus pressure was in part due to a stronger counterregulation on the part of the aortic baroreflex However if the finding in dogs that this reflex is relatively ineffective in buffering a decrease in arterial pressure below normal (Donald and Edis 1971 Pelletier Clement and Shepherd 1972) also pertains to man this discrepancy in blood pressure response to changes in transmural sinus pressure to either side of the normal would reflect important characteristics of the carotid sinus mechanism in man These characteristics differ from those obtained in anesthetized vagotomized animals with surgically exposed and isolated open loop carotid sinus preparations which have shown nearly symmetrical blood pressure responses to changes in mean intrasinus pressure on either side of the normal (for references see Heymans and Neil 1958) Intrasinus pulse pressure and dp/dt which may interact non additively with the changes in intrasinus mean pressure as determinants of baroreceptor activity (for references see Folkow and Neil 1971 Zoller *et al* 1972) may also have contributed to the observed nonlinearity of the system by exerting different effects at different levels of mean transmural pressure

The present experiments were directed toward studying the relative importance of the cardiac and peripheral effector systems in the buffer function of the carotid sinus baroreceptors Measurements of cardiac output showed a significant 10% increase when the transmural sinus pressure was lowered in the supine and head up position whereas no significant change occurred with a corresponding increase in transmural pressure Thus the difference between the magnitudes of the blood pressure responses in the two cases is attributable to differences in the contributions of cardiac output and systemic vascular resistance whereas in both body positions the arterial pressure response to increased transmural sinus pressure was due solely to a reduction in vascular resistance a significant increase in cardiac output contributed to the more potent pressor response to a decrease in transmural pressure and thus to the predominantly antihypotensive properties that characterize the carotid sinus baroreceptor control system in man The observation that a subatmospheric pressure of 40 mm Hg over the neck did not influence cardiac output is in agreement with the results of Ernsting and Perry (1957) Bevegård and Shepherd (1966) on the other hand reported a decrease in cardiac output in six subjects with subatmospher pressures of -0 to −60 mm Hg, but this effect was statistically significant onl mm Hg The present data *indicate* that reflex counter regulation of cardiac o is more easily elicited by a decrease than by an increase in transmural sinus

Combined beta adrenergic and parasympathetic blockade strongly influenced the cardiovascular adjustments following a change from the supine to the head up position Thus, from the control values for cardiac output given in Table II it is evident that a shift from the supine to the head up position caused a much greater fall in this variable after blockade than it did before (51 and 26% respectively $p < 0.05$) In spite of this mean arterial pressure in the head up position was not lowered and the exaggerated curtailment of cardiac output after blockade was therefore fully compensated by a greater increase in systemic vascular resistance The question remains whether the greater fall in cardiac output following autonomic blockade was due to cardiac or peripheral effects or both Calculated average stroke volume in the head up position was smaller after than before blockade (48 and 67 ml respectively $p < 0.01$) presumably in part due to blockade of beta adrenergic receptors which has previously been shown to diminish stroke volume in the sitting (Bjurstedt *et al* 1974) and standing (Sannerstedt Julius and Conway 1970) positions Loss of baroreceptor induced sympathetic inotropic drive to the heart may have been involved (*cf* Sarnoff *et al* 1960 Gli k 1971) but decreased venous return may also have contributed secondary to increased blood pooling in infracardiac capacitance vessels by the action of atropine (Kalser Frye and Gordon 1954 Miller *et al* 1954) and by prevention of the venoconstriction normally mediated by stimulation of beta adrenergic receptors (*cf* Kaiser Ross and Braunwald 1963)

It can be seen in Table III that the difference in magnitude between the blood pressure responses to increased and decreased external pressure on the neck both in the supine and head up positions was less evident during combined beta adrenergic and parasympathetic blockade which prevented any intervention on the part of the cardiac effector mechanism. That in the absence of such intervention the reflex blood pressure responses to changes in transmural sinus pressure were not greatly impaired however indicates that baroreceptor induced adjustments in cardiac output are not of critical importance in the buffer function of the carotid sinus baroreceptors This is concordant with the observation of Epstein *et al* (1969) that electrical stimulation of the carotid sinus nerve in man causes a fall in systemic arterial pressure also when cardiac output is kept constant by mild exercise The present finding that the baroreceptor induced blood pressure response was not greatly influenced by either posture or autonomic blockade also tallies with other results obtained with electrical stimulation of the sinus nerve in man (Eckberg *et al* 1972)

This work was supported b gr s fr m the Swed sh Medical Research Council (Project No 650) and Gösta Fraenckel s Fund

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Acta physiol scand 1975 94 506–516

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Vasomotor Nerve Control of Isolated Arteries and Veins

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Received 6 February 1975

Abstract

LJUNG B., J A BEVAN B L PEGRAM R E. PURDY and M SU *Vasomotor nerve control of isolated arteries and veins* Acta physiol scand 1975 94 506–516

In order to compare neuro-effector function in different blood vessels, frequency response relationships were determined for the following preparations 1) Isolated rings of the proximal saphenous, distal saphenous and ear arteries, the parietal branch of the internal iliac vein and the small saphenous vein from the rabbit, 2) spiral strips of the rabbit pulmonary artery and 3) longitudinal preparations of the rat portal vein In each rabbit tissue only one low (4 Hz) and one high (8 Hz) transmural nerve stimulation frequency was applied until steady state responses were obtained and these were expressed as a percentage of a maximum response to exogenous noradrenaline (NA) applied in each experiment The general shape of the frequency response curves was similar but differences in steepness and amplitude of the maximum neurogenic response relative to exogenous NA were found. The steepness of the frequency response relationships of the veins tended to be greater than those of the arteries. It appears that factors such as close neuro-muscular contacts presence of terminal nerve fibres within the media and the operation of mechanisms for myogenic propagation of activity contribute to the effectiveness of neurogenic vascular control as revealed by frequency response curves. *In vivo* geometrical factors can greatly augment the hemodynamic significance of the observed differences.

The predominant extrinsic control of the vascular smooth muscle in most vascular beds is exerted *via* postganglionic adrenergic nerve fibres During the last decade neurogenic vascular control has been analyzed in some detail in a select number of isolated blood vessel preparations (for ref see Bevan and Su 1973) Large arteries and veins are primarily suited to this type of study for technical reasons

There is considerable variation in the extent and characteristics of nervous control of the hemodynamically important small arteries and veins *in vivo* For example Mellander (1960) found that venous capacitance responses to vasomotor nerve stimulation at low impulse rates were relatively more pronounced than arterial resistance responses in the

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hindquarter of the cat Variation in the nervous control of arterial resistance has also been found in different vascular circuits (for ref see Mellander and Johansson 1968) Such quantitative inequalities *in vivo* are difficult to evaluate in terms of smooth muscle activation because of the complex interrelations and geometry of consecutive vascular sections

In order to directly compare frequency response relationships in arterial and venous smooth muscle isolated ring preparations of different arteries and veins from the rabbit have been studied in the present experiments Isometric force responses to electrical field stimulation at graded impulse rates were determined in the proximal saphenous distal saphenous ear and pulmonary arteries the parietal branch of the internal iliac vein and the small saphenous vein Differences in the frequency dependence and maximum extent of the adrenergic nervous control of these vessels seem to be related to previously reported variation in their neuro-effector organization

Methods

A bino rabbits weighing 2 0–4 5 kg were stunned by a blow to the head and exsanguinated The following blood vessels were carefully isolated dissected under a dissection microscope and placed in a bath of oxygenated Krebs bicarbonate solution the central ear artery (proximal part) the pulmonary artery the proximal saphenous artery (at the thigh) the distal saphenous artery (above the ankle) a parietal branch of the internal iliac vein and the small saphenous vein (below the knee joint) Ring preparations were made of most vessels by cutting them in sections 3–5 mm in length These rings were mounted under 5 10 mN passive force for recording of isometric responses according to the method described by Bevan and Osher (1972) For technical reasons spiral strip preparations were made of the pulmonary artery instead of rings

Results from the rat portal vein previously reported in preliminary form (Ljung 1970) have been included as well for comparison The experimental method for isolation and mounting of the longitudinal muscle preparation of the rat portal vein has been described elsewhere (see Ljung 1970)

Nerve responses were obtained by selective activation of the intramural adrenergic nerve plexus by means of transmural electrical field stimulation A Grass S-4 stimulator provided square wave impulses of supramaximum amplitude and 0 3–0 8 ms in duration to platinum electrodes mounted on either side of the preparation Frequency response relationships were obtained in experiments performed according to the following protocol The tissue was first allowed to accommodate for 1 h Transmural field stimulation was then applied at a low frequency of either 0 5 1 or 4 Hz until a plateau was reached After a resting period of 15 min a second stimulation at an impulse rate of 8 Hz or higher was similarly made to a plateau level When the baseline had completely returned to the resting level of passive force (usually 15 min later) a maximum response to exogenous noradrenaline (NA) was induced by injection of NA 10^{-4} M into the bath solution In this way series of experiments on preparations from a certain blood vessel provided an accurate compounded frequency response curve where each preparation contributed one equilibrium response value from the low (4 Hz) and one from the high (4 Hz) impulse frequency ranges Plateau values for isometric force were expressed as a percentage of the maximum NA response and plotted against impulse frequency In addition the mean responses to each impulse frequency were expressed as a percentage of the maximum response to nerve stimulation in each series of experiments on a particular blood vessel

Concentration effect curves for exogenous NA were obtained in three selected vessels strip preparations of the pulmonary artery and ring preparations of the ear artery and small saphenous vein by injections of NA in increasing concentrations into the bath for 3 min at 15 min intervals with repeated washings in between The NA response values were expressed as a percentage of the maximum NA response The agonist concentration which gave half the maximum response (EO_{50}) was determined graphically in each experiment In separate experiments the NA sensitivity was determined after blockade of the neuronal uptake by desmethylimipramine (DMI 10 M) and after blockade of the β adrenergic receptors by propranolol (10 M)

Comparison of mean values was performed by Student's t test, pairing design or by means of analysis

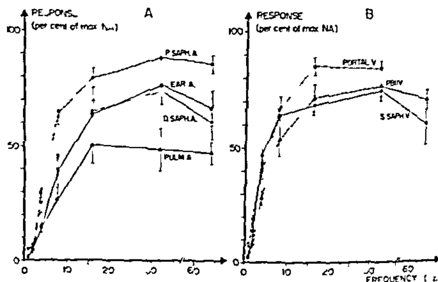


Fig. 1 Frequency response relationships obtained in isolated arteries (A) and veins (B) from the rabbit. Each preparation stimulated with one low (< 4 Hz) and one high impulse frequency only. Responses expressed as a percentage of a maximum response to NA. Number of preparations. Proximal saphenous artery (P SAPH.A.) 8 ear artery 56 distal saphenous artery (D.SAPH.A.) 78 pulmonary artery (PULM A.) 35 parietal branch of internal iliac vein (PBIIV) 56 and small saphenous vein (S SAPH V) 56. For comparison a curve obtained in 20 rat portal vein preparations stimulated in random order (Ljung 1970) is included in Fig. 1B. Mean values ± S E.

Results

Curves for the various blood vessels relating nerve stimulation frequency to the contractile response amplitude expressed as a percentage of maximum noradrenaline (NA) response (10^{-4} M) are illustrated in Fig. 1. Their general configuration is similar in that they are steep in the low frequency range (1–4 Hz) of postganglionic sympathetic nerve stimulation and level off to reach maxima at 16–32 Hz. It is of interest to compare the amplitudes of the maximal neural responses and the steepness of the initial segments of these curves. The variation in the former parameter (Table I) is apparent from Fig. 1. Of the arteries studied (Fig. 1 A) the proximal saphenous artery gave the greatest neurogenic relative to the NA response and the pulmonary artery the smallest. The amplitudes for the distal saphenous and the ear arteries ranged in between these values and so did those of both the small saphenous vein and the parietal branch of the internal iliac vein (Fig. 1 B). Corresponding data for the longitudinal smooth muscle of the rat portal vein (Ljung 1970) have also been included in Fig. 1 B and Table I. Its maximum neurogenic response value was as high as that of the proximal saphenous artery.

In Fig. 2 the mean responses to each impulse rate have been expressed as a percentage of the maximum neurogenic response of a particular vessel. Although the shapes of the curves for the different arteries and veins are similar, their initial slopes do vary, i.e. the sensitivity to changes in impulse rate within the low frequency range is different. In order to quantitate these differences two findings from Fig. 2 were utilized. First, threshold stimulation frequencies were found to be essentially the same in the different vessels and

TABLE 1 Responses of isolated arteries and veins to transmural stimulation

Blood vessel	Maximum response		
	Amplitude (max. NA±S E.)	f_{00}[a] (Hz)	f_{50} (Hz)
Pulmonary artery	51±8 1	16	14 3
Dist saphenous artery	77±3 5	3[illegible]	10 3
Ear artery	74±5 8	32	8 5
Prox saphenous artery	88±5 5	32	5 5
Parietal branch of internal iliac vein	77±4 5	32	7 4
Small saphenous vein	75±4 4	32	4 [illegible]
Rat portal vein	85±3 8	16	4 9

[a] f_{00} and f_{50} indicate the frequencies at which maximum and half maximum neurogenic responses were obtained *respectively* f_s *was determined by linear regression*

secondly the relationship between impulse rate and response amplitude was practically linear for responses of up to 60–70 per cent of the maximum neurogenic response (Fig 2) Therefore the value of the frequency which gave 50 per cent of the maximal neurogenic response f_s could be approximately determined by linear regression analysis and taken to represent the steepness of the frequency response curves The obtained f_s values (Table I) were lowest for the small saphenous vein and the rat portal vein and twice as high in the distal saphenous and pulmonary arteries The frequency response curves of the veins tended to be steeper than those of the arteries This distinction did not apply to the maximum response values Thus it seems that the extent and characteristics of the neuro-effector control in a particular blood vessel is determined by the functional properties of its vasomotor nerve supply and smooth muscle (see Discussion)

The pulmonary and ear arteries and the small saphenous vein were selected for study of the sensitivity of the smooth muscle to exogenous NA in separate expts The shape and

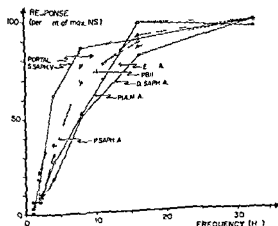


Fig — Frequency response curves of different arteries and veins Mean values from Fig. 1 expressed as a percentage maximum response to nerve st

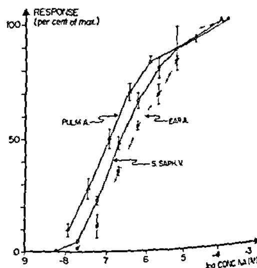


Fig 3 Concentration-effect curves for nor adrenaline (NA) obtained in experiments on strip preparations of pulmonary artery (n = 8) and in ring preparations of the ear artery (n = 4) and the small saphenous vein (n = 4) Note that in ring preparations NA will enter the tissue via the adventitial surface only

slope of the concentration-effect curves (Fig 3) were comparable in the three vessels. The apparent sensitivity to NA was expressed as that concentration which produced half the maximum response ED_{50} and found to be of the same order of magnitude for all three vessels in the control situation (Table II) Blockade of the neuronal uptake mechanism, by treatment with desmethylimipramine (DMI 10^{-7} M) caused a 13 fold decrease in ED_{50} for the ear artery but the ED_{50} value was only reduced by a factor of 2 9 and 1 8 in the small saphenous vein and in the pulmonary artery respectively It thus seems that the sensitivity of the effector tissue proper to excitation by NA is greater in the ear artery than in the two other vessels Blockade of the β adrenergic receptors with propranolol (10^{-7} M) did not significantly alter the ED_{50} values (Table II)

Discussion

lood vessels must react to meet varying functional demands within the consecutive sections of the circulatory system and in the parallel-coupled circuits of different organs (see Mellander and Johansson 1968 Folkow and Neil 1971) In a recent study Gillespie and Rae (1972) showed that the extent of neurogenic response of isolated perfused arteries is related to their innervation density and wall to lumen ratio In veins the isometric force response

TABLE II Sensitivity to exogenous NA ED_{50} 10^{-7} M

Blood vessel	Control	DMI 10^{-7} M	Control DMI 10^{-7} M	Propranolol 10^{-7} M + DMI
Pulmonary artery (n = 8)	1 8 0 37	1 1 0 [illegible]		
Ear artery (n = 4)	4 9 0 64	0 39 0 11	0 45 ± 0 05	0 53 ± 0 06
Small saphenous vein (n = 4)	[illegible]7 0 0	0 94 ± 0 18	1.5 ± 0 53	1 7 ± 0 64

to nerve stimulation may be correlated primarily with the supply of adrenergic nerves (Bevan *et al* 1974 a b)

In the present experiments the amplitude of the maximum neurogenic responses relative to their own maximum responses to exogenous NA (Fig. 1 Table I) did not fall into artery and vein groupings However the veins tended to respond relatively more than the arteries to stimulation at low impulse rates as reflected by steeper initial segments of the frequency response curves (Fig 2 Table I)

In different blood vessels considerable variation has been found to occur in such factors as density and distribution of the terminal adrenergic plexus width of neuro-muscular separations mechanisms for transmitter elimination and properties of the effector tissue (for ref see Bevan and Su 1973) The present experiments were performed to make a comparison possible between frequency response relationships of some isolated arteries and veins In the following the results will be discussed in relation to available information about their neuro-effector organization

The pulmonary artery gave the lowest maximum neurogenic response Furthermore a high impulse rate (14 Hz) was required to elicit half the maximum response In rabbit elastic arteries very wide clefts prevail between the adrenergic plexus at the adventitio-medial junction and the nearest smooth muscle cells (Verity and Bevan 1968) The deeper parts of the media become activated as the transmitter diffuses into succeeding muscle layers since myogenic spread of activity does not seem to occur (Bevan *et al* 1970) As a consequence the transmitter is distributed within a large fluid compartment and the mean effective concentration in the media is comparatively low (Bevan and Su 1974) The amplitude of the maximum isometric response to nerve stimulation and the steepness of the frequency response curve would be determined primarily by the mean NA concentration attained within the entire muscle layer during nerve activity at the different impulse rates It may be that a failing transmitter output at high impulses rates (*e g* Brown and Gillespie 1957 Kirpekar and Misu 1967 Davies and Withrington 1968 Haggendal *et al* 1970) at least in part sets the level of the maximal neurogenic response in some large vessels and causes the decreased responses found at supramaximal impulse rates

In the rat portal vein a commonly used model for *in vitro* studies of spontaneously active vascular muscle the neuro-muscular separation (*cf* Booz 1971) is more than one order of magnitude less than in the elastic arteries of the rabbit There is evidence that a functional neuro-muscular junction exists in this vessel (see Ljung 1970) High but short lasting transmitter concentration peaks induce excitation of the muscle cells next to the nerve terminals from where the activity seems to be propagated myogenically throughout the media The frequency response curve of the rat portal vein (Ljung 1970) included in Fig. 1 B was steep in the initial segment and showed one of the greatest response values to nerve stimulation According to a quantitative model analysis of the neuroeffector function in the portal vein (Johansson *et al* 1972) the shape of the curve and the amplitude of the maximal neurogenic response relative to the exogenous NA response are greatly influenced by postjunctional mechanisms in that vessel

The pattern of the neuro-effector function of the rat portal vein is essentially diff from that which pertains to elastic arteries. However in spite of the two different pr

that appear to govern adrenergic vasomotor control of the rabbit pulmonary artery and the rat portal vein frequency response curves of similar configuration are obtained in the two vessels (Fig 2)

It is likely that the rabbit elastic arteries and the rat portal vein respectively represent rather extreme variants in a spectrum of vascular neuro muscular arrangements (see Bevan and Su 1973 Ljung 1970) This assumption is supported by the present finding that the maximum neurogenic responses of all the muscular arteries except the proximal saphenous artery and of the peripheral veins ranged intermediately between the extremes of these two vessels In the proximal saphenous artery the adrenergic terminal nerve structures form a dense network at the adventitio medial junction and in addition they do enter the outer media whereas the distal section of the same artery receives only a two-dimensional nerve plexus (Bevan and Purdy 1973) The maximum neurogenic response of this vessel was significantly smaller and the frequency response curve was less steep than its proximal counterpart (Fig 1 2 Table I) It is suggested that the great maximal neurogenic response and the high sensitivity to transmural nerve stimulation in the low frequency range found in the preparation with medial innervation (Fig 1 2 Table I) is related to the shorter distances required for transmitter diffusion and/or myogenic spread of neurogenically induced activity

The small saphenous vein also has adrenergic innervation inside the medial layer (Bevan *et al* 1974 b) This thin walled cutaneous vein often showed phasic activity superimposed upon the tonic nerve induced responses which demonstrates the existence of a presumably myogenic coordinating mechanism (*cf* Johansson and Ljung 1967) Since longitudinal propagation of activity occurs in small peripheral arteries such as the posterior tibial artery (Bevan and Ljung 1974) it seems likely that myogenic mechanisms may contribute to the recruitment and coordination of medial cells in the response of small arteries and veins

The maximum neurogenic response of the parietal branch of the internal iliac vein was comparable to that of the small saphenous vein The former vessel is supplied with a two-dimensional plexus (Bevan *et al* 1974 b) the density and NA uptake capacity of which is slightly less than that of the small saphenous vein Tissue sensitivity to exogenous NA extra neuronal NA uptake and activity of NA catabolizing enzymes seem comparable in the two vessels (Bevan *et al* 1974 a) The observed difference in steepness of the frequency response relationships of these two veins (Fig 2 Table I) thus might be the consequence of differences in the distribution of the nerve terminals

In the present experiments the maximum neurogenic response of the ear artery of the rabbit which receives a heavy [illegible] innervation was 75 per cent of the NA response and thus was comparable to the [illegible] saphenous artery and the two peripheral veins (Table I) The sensitivity to exogenous NA was greater in the ear artery than in both the pulmonary artery and in the small saphenous vein after blockade of the neural uptake mechanism (Table II) This is in accordance with a previously noted tendency for the NA ED_{50} values of various venous effector tissues [illegible] ersely related to the density of innervation (Bevan *et al* 1974 b) Thus it appears th[illegible] scular smooth muscle from the same species but from different blood vessels exhibits variation in NA sensitivity which may be correlated with the dimensions of the vasomotor nerve supply The significance of this finding awaits further experi

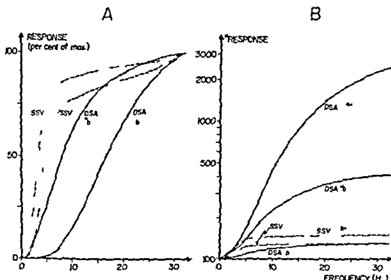


Fig 4 Frequency response curves of small saphenous vein (SSV) and distal saphenous artery (DSA) In A curves a and b correspond to the curves in Fig. 2. a and b^4 represent the relationship for the square and fourth power responses respectively when expressed as a percentage of max response In B "responses" in absolute terms have been expressed as a percentage of control as uming that maximal activation of the muscle corresponds to shortening by 30 per cent. Note the pronounced change in resi tance expected to occur in the artery (r_i^4) assuming a wall to lumen ratio of 1/5 as compared to the capacitance response (a) in the vein for the same degree of smooth muscle shortening

mental analysis The dense adrenergic plexus of the ear artery also explains the pronounced prejunctional supersensitivity (Table II) obtained when exogenous NA is applied via the adventitial surface after elimination of the neuronal NA uptake mechanism (de la Lande Frewin and Waterson 1967) In the present experiments DMI reduced the ED_{50} value by a factor of 13 in the ear artery as opposed to a value of 3 and 2 in the small saphenous vein and the pulmonary artery respectively (Table II)

The frequency response curve of the ear artery ranged in between those of the pulmonary artery and the rat portal vein with regard to steepness and maximal response (Table I) but was comparable to most of the curves for the peripheral vessels studied Thus it can be concluded that in spite of an extraordinarily heavy terminal nerve plexus and a comparatively high effector sensitivity to exogenous NA the frequency response characteristics in the ear artery are similar to those in other vessels of corresponding calibre

On the whole the present results demonstrate that the general configuration of curves relating isometric force responses to nerve stimulation impulse frequency is similar for a variety of isolated rabbit vessels However differences do exist particularly in the steepness of the initial part of the curve The presence of nerve terminals within the media and myogenic propagating mechanisms are apparently of importance

The postganglionic sympathetic fibres to blood vessels normally discharge at low rates and even under intense reflex activation tonic impulse rates rarely exceed 6–8 Hz kow 1952, Kendrick Öberg and Wennergren 1972) Thus the important part quency response curve must be the initial segment, where the major diff

vessels also seem to occur (*cf* Fig 1) *In vivo* cardiovascular responses will not be linearly related to effector response and some consequences of this fact are illustrated in Fig 4 The frequency response curves of the small saphenous vein (curve a) and the distal saphenous artery (curve b) from Fig 2 are taken to represent smooth muscle activation If the muscle had also been allowed to shorten like in the physiological situation then presumably the curves would have been steeper (*cf* Rosenblueth 1932) The curves of these two cutaneous vessels from the same section of the rabbit s hind limb actually displayed the greatest and the smallest curve steepness respectively *In vivo* the functional response of the vein would be to reduce its content of blood a parameter proportional to the square of the smooth muscle shortening whereas the resistance function of an artery would be related to the fourth power of the smooth muscle contraction The curve relating the square (a^2) and the fourth power (b^4) of the response values to impulse frequency are displaced to the right to different extents

Mellander (1960) found frequency response relationships *in vivo* for capacitance and resistance vessels in the hind quarter preparation which clearly resembled curve a^2 and b^4 (Fig 4 A) respectively when studied under conditions of constant arterial inflow pressure and venous outflow pressure However in another report Browse Lorenz and Shepherd (1966) found identical characteristics for the corresponding two frequency response relationships when capacitance responses of the dog s occluded hind lib were compared to those of the resistance vessels perfused at constant flow The differences between the results of these two studies have been attributed to methodological factors (Mellander and Johansson 1968) The variation in frequency response characteristics of isolated arteries and veins, albeit from larger vessels found in the present study in conjunction with the above mentioned influence of different mathematical power functions renders it less likely that similar frequency response relationships would prevail in the resistance and capacitance sections of a vascular bed *in vivo*

Mellander (1960) stressed that the vasoconstrictor effects on the venous compared to the arterial sections were more pronounced only when frequency response relationships were expressed as a percentage of the maximum neurogenic response The present results could be utilized to compute approximate capacitance and resistance "responses" of the small saphenous vein and the distal saphenous artery if it is assumed that the isometric force responses could be taken to represent smooth muscle shortening If it is further assumed that the [illegible]imal response [illegible] both tissues correspond to a decrease by 10 per cent of the outer vasc[illegible] radius the fre[illegible]y response curves would be represented by a and b in Fig 4 B The [illegible] of the small saphenous vein would be related to the change in cross-sec[illegible]nal are[illegible] and the change in resistance of the artery would accordingly be inver[illegible]ly relate[illegible] [illegible]th power of the change in vascular radius (curve b) [illegible]

The wall to lumen ratio [illegible] known to be of great importance in determ[illegible] hemodynamic effects [illegible] e wall thickness of the distended small saphenous vein can be disregarded but th[illegible] f wall thickness to lumen radius is 1:5 in the distal saphenous artery (*cf* Bevan a[illegible] 973) As a result the "resistance" curve [illegible] which relates the change in the fourth [illegible] f the internal radius to impulse rate [illegible] become greatly augmented

In spite of the approximations involved the calculated maximal values which thus correspond to a reduction of venous blood content by 50 per cent and an increase in arterial flow resistance by a factor of 10–20 are of the order of magnitude found *in vivo* (*cf* Mellander 1960) It is thus evident that seemingly small differences in the characteristics of frequency response relationships of isolated blood vessels due to variation in neuro-effector properties can be of great hemodynamic significance in view of the augmentation of effects derived from geometrical factors

Supported by US Public Health Service grant H2 08359 Los Angeles County Heart Association grant 408 I G and by the Swedish Medical Research Council 3884

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Acta physiol scand 1975 94 517–521

From the Institute of Physiology University of Lund Sweden

Stimulation of Adrenergic Nerve Fibres to the Urinary Bladder of the Rat

By

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Received 24 February 1975

Abstract

ELMER, M *Stimulation of adrenergic nerve fibres to the urinary bladder of the rat* Acta physiol. scand 1975 94 517–521

The contraction of the rat detrusor muscle caused by electrical stimulation of the hypogastric or pelvic nerves was followed by relaxation when the nerves were stimulated for a short period. The relaxation was more pronounced when the initial contraction was reduced by atropine It was found to be mediated by adrenergic fibres acting on inhibitory β receptors. Stimulation of the hypogastric or pelvic nerves at high frequencies increased the contractile response probably via adrenergic fibres activating excitatory α receptors

Key words Urinary bladder nerve stimulation, adrenoceptors

In pharmacological experiments the presence of both excitatory α adrenoceptors and inhibitory β-receptors in the detrusor muscle of the rat has been shown (Elmer 1974 a) and histochemically adrenergic as well as cholinergic nerve fibres innervating the bladder have been detected in both the hypogastric and pelvic nerves (Alm and Elmer 1975)

In the present investigation the bladder responses to stimulation of adrenergic fibres in the hypogastric and pelvic nerves were studied

Methods

I male albino rats of the Wistar strain weighing about 350 g were used.

The rats were anesthetized with chloralose (100 mg/kg) given through a cannula in a femoral vein after induction with ether The bladder was exposed and the ureters were ligated A glass cannula was inserted into the bladder through an incision in the urethra The bladder was filled with 0.5 ml of physiological saline and the pressure developed by the detrusor muscle was recorded by means of a transducer and a polygraph

The hypogastric nerves were cut distal to the hypogastric ganglia and the distal ends were stimulated jointly using a bipolar electrode Each pelvic nerve was stimulated after section proximal to the pelvic plexus, which in the male rat forms a distinct ganglion located on the lateral surface of the prostate gland (Langworthy 1965). 3 Grass stimulators supplied with stimulus isolation units giving rectangular pulses with a duration of ms, a frequency of 0 1 200 Hz and of supramaximal intensity (10 V) were used.

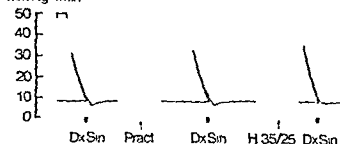


Fig. 1 Pressure responses of the rat urinary bladder to electrical stimulation of the pelvic nerves bilaterally at [illegible]0 Hz (Dx Sin) after the injection of atropine 1 mg/kg and dihydroergotamine [illegible] mg/kg, Pract the β_1 blocking agent practolol 10 mg/kg, H 35/25 the β_2 blocking agent H 35/25 1 mg/kg

For statistical evaluation of the data Student's t test for paired samples was used The 0.05 level of probability was accepted as significant.

Drugs The substances used were hexamethonium bromide atropine sulphate guanethidine bisulphate dihydroergotamine methansulphonate propranolol hydrochloride practolol and H 35/25 (*1-isopropyl amino-1-(4 methylphenyl) etanol*) The drugs were injected through the cannula in the femoral vein.

Results

Electrical stimulation of the hypogastric or pelvic nerves at 20 Hz for a period of 15 s caused contraction of the detrusor muscle increasing the intravesical pressure from a resting pressure of about 10 mm Hg. When the nerves were stimulated for 5 s the contraction was followed by relaxation in 5 out of 9 rats After the injection of atropine 0.1–1 mg/kg, the contraction of the bladder was reduced by 60[illegible] and was always followed by relaxation when the nerves were stimulated for a period of 5 s (Fig. 1) When the stimulation period was longer the relaxation was probably masked by predominant contraction in spite of the fact that the contractile response was reduced after atropine Maximal relaxation was obtained with stimulation frequencies between 1 and 20 Hz decreasing the intravesical pressure by 0.5–4 mm Hg The inhibitory response to stimulation of the pelvic nerves at 20 Hz was 1.8 ± 0.4 mm Hg (mean ± S.E. n = 9) and the hypogastric nerves 1.0 ± 0.3 mm Hg ($n = 5$)

The inhibitory responses were not affected by previous injection of dihydroergotamine 2 mg/kg or the β_1 adrenoceptor blocking agent practolol 10 mg/kg but were totally abolished by guanethidine 1 mg/kg propranolol 2–4 mg/kg or the β_2-blocking agent H 3[illegible]25 1 mg/kg (Fig. 1)

The contraction of the detrusor muscle caused by stimulation of the hypogastric nerves reached a maximum at stimulation frequencies of 15–20 Hz. At 30 Hz the response was

TABLE 1 Pressure responses in mm Hg of the rat urinary bladder to electrical stimulation of the hypogastric nerves bilaterally (H), the pelvic nerves unilaterally (Dx or Sin) and the pelvic nerves bilaterally (Dx Sin). Values are mean ± S.E. n = number of experiments. p = significance at paired comparisons.

	H	Dx or Sin	Dx Sin
20 Hz	2.1 ± 0.5	18.9 ± .6	58.6 ± 6.8
50–100 Hz	5.1 ± 1.5	41.8 ± 7	61.9 ± 5.9
n	8	14	7
p	0.05	0.001	< 0.1

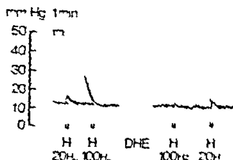


Fig. – Pressure responses of the rat urinary bladder to electrical stimulation of the hypogastric nerves (H) before and after the injection of dihydroergotamine mg/kg (DHE)

somewhat lower but when the nerves were stimulated at higher frequencies the contractile response increased again, reaching another maximum which was 1–10 mm Hg higher at stimulation frequencies of 50–100 Hz. When the pelvic nerves were stimulated unilaterally using increasing frequencies the responses reached a plateau at 15 Hz and remained unchanged until the frequency was raised to 40 Hz. Then the response increased by 1–6 mm Hg to a maximum at 50–100 Hz. The response to bilateral stimulation of the pelvic nerves was not significantly changed by stimulation at these high frequencies (Table I)

The increased part of the responses caused by the high stimulation frequencies was not affected by previous injection of hexamethonium 10 mg/kg atropine 1 mg/kg or propranolol 2 mg/kg but was totally abolished by guanethidine 1–3 mg/kg or dihydroergotamine 2 mg/kg (Fig 2)

Discussion

The contractile response of the rat bladder to stimulation of the pelvic nerves at 15–50 Hz is probably caused via cholinergic fibres (Carpenter and Rand 1965 Chesher 1967 Elmér 1973 1975) and this seems also to be the case at stimulation of the hypogastric nerves in the guinea pig (Mantegazza and Naimzada 1967) and in the rat (Alm and Elmér 1975 Elmér 1975) These responses to hypogastric or pelvic stimulation in the rat are not affected by adrenoceptor blocking agents but reduced by about 60 % after atropine and potentiated after eserine

In dogs and cats some authors have found a relaxation of the bladder after an initial contraction when stimulating the hypogastric nerves (Griffiths 1895 Kuntz and Saccomanno 1944) The contraction in the cat is probably caused by activation of excitatory α adrenoceptors and the relaxation by inhibitory β-receptors (Edvardsen 1968) The relaxation is more protracted than the contractile response similar to the relaxation of the rat bladder found in this study when the hypogastric or pelvic nerves were stimulated for a short period and the contractile response was reduced by atropine

The relaxation of the detrusor muscle found in the present investigation when the pelvic or hypogastric nerves were stimulated after atropine was abolished by the adrenergic neurone blocking drug guanethidine indicating that the relaxation was caused by stimulation of the adrenergic fibres demonstrated histochemically in both nerves (Alm and Elmér 19[illegible] existence of two types of β-adrenoceptors was suggested by Lands *et al* (196[illegible] heart and small intestine and β in the bronchi blood vessels and uterus

experiments have shown that the inhibitory β adrenoceptors of the rat urinary bladder belong to the type of receptors classified as β_2-receptors (Elmér 1974 b) The β_1 receptor blocking compound practolol (Dunlop and Shanks 1968) did not affect the inhibitory bladder response to nerve stimulation in the present study when given in a dose which completely blocks the β_1 receptors of the heart in the cat (Åblad *et al* 1973) The relaxation of the bladder was however totally abolished by the non selective β adrenoceptor blocking agent propranolol or the β_2-blocking agent H 35/25 (Levy 1967 Levy and Wilkenfeld 1969 Carlsson *et al* 1972, Johansson 1973) indicating that the inhibitory effect of adrenergic nerve stimulation is exerted by action on β_2-receptors

The increased part of the contraction of the detrusor muscle obtained when one of the pelvic nerves or the hypogastric nerves were stimulated at high frequencies was not affected by hexamethonium atropine or propranolol but was abolished by guanethidine or dihydroergotamine suggesting that postganglionic adrenergic fibres were stimulated acting on α receptors The presence of excitatory α adrenoceptors in the detrusor muscle of the rat has been shown in pharmacological experiments (Elmér 1974 a) Garry and Gillespie (1955) found the same high stimulation frequency to be optimal for stimulation of the sympathetic nerves to the rabbit colon

Hypothetically the explanation of the finding that the α receptors were not activated at low stimulation frequencies but only at these high frequencies might be an overflow of transmitter from the adrenergic fibres shown to be present in the detrusor muscle partly around blood vessels (Alm and Elmér 1975) activating α receptors in muscle fibres not innervated by adrenergic nerves The adrenergic innervation of the rat detrusor is sparse and the terminal fibres are not associated with individual muscle cells but only with large groups of muscle fibres (El Badawi and Schenk 1966 Alm and Elmér 1975) The assumption that the excitatory α receptors are activated when the amount of transmitter released is increased is in agreement with the results of Edge (1955) who obtained a bladder contraction in the cat by intraarterial injection of noradrenaline but relaxation after equivalent doses intravenously Two weeks after denervation of the rat bladder the response to injected noradrenaline is changed from relaxation to contraction probably because of sensitization of α receptors more than β-receptors (Elmér 1974 a) Six to ten weeks after parasympathetic denervation an outgrowth of new adrenergic terminals in the detrusor muscle of the cat is observed changing the normal inhibitory β-receptor response to hypogastric nerve stimulation to an excitatory α receptor response (Sundin and Dahlström 1973) Thus existing but not utilized α receptors might be supposed to be activated postulating that these receptors demand a higher concentration of noradrenaline than do the β-receptors

This work was supported by [illegible] from the Medical Faculty University of Lund
H 35/25 was kindly supplied [illegible] AB Hässle Göteborg.

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Acta physiol scand 1975 94 522–535
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Characterization of the Metabolism of Exogenous Cyclic AMP by Perfused Rat Heart and Incubated Prepubertal Rat Ovary

By

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Received 6 March 1975

Abstract

ROSBERG S G SELSTAM and O ISAKSSON *Characterization of the metabolism of exogenous cyclic AMP by perfused rat heart and incubated prepubertal rat ovary* Acta physiol scand 1975 94 522–535

In order to study the metabolism of extracellular 3',5' adenosine monophosphate (cAMP), rat hearts were perfused and prepubertal rat ovaries incubated with ^3H and ^{32}P labelled cAMP (0.0.5 1 μM). The rate of disappearance of cAMP from the medium was determined by "Ba Zn precipitation" and degradation products of ^3H and ^{32}P-cAMP by paper chromatography Both tissues degraded cAMP to 5' adenosine monophosphate (AMP) but the enzyme kinetic for this phosphodiesterase activity was different (apparent K_m value for the heart 3.95 μM and for the ovary 0.2 μM). AMP was further degraded, since also other labelled substances were found in the medium. An uptake of both ^3H and ^{32}P labelled substance(s) into the heart and the ovary was noticed Tissue extracts contained several labelled purines, but the amounts of labelled cAMP did not exceed expected amounts in the extracellular space In the ovary the uptake of cAMP and AMP seemed to be low since the uptake of labelled substances was [illegible] by high concentrations of unlabelled AMP or adenosine The degradation of ^{32}P-cAMP was unchanged when AMP was present, strongly suggesting that the phosphodiesterase enzyme was acting extracellularly. In the heart added AMP was very rapidly degraded making it impossible to elucidate whether cAMP was degraded extracellularly or not It is concluded that elimination of extracellular cAMP under physiological conditions can be due to degradation of cAMP by various tissues At least for the ovary this phosphodiesterase enzyme is extracellularly active

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Cyclic 3',5' adenosine monophosphate (cAMP) is released from many tissues in response to hormonal stimulation as has been shown in studies both *in vivo* and *in vitro* (Davoren and Sutherland 1963 Exton *et al* 1972, Cramer and Lindl 1974 Franklin and Foster 1973 Peytremann *et al* 1973 D[illegible] *et al* 1973 Selstam *et al* 1974) The magnitude of the release differs markedly between different types of tissues In some tissues *e g* the perfused liver and the incubated prepubertal rat ovary a release of cAMP in response to low concentrations of hormones is measurable even without a detectable intracellular elevation of cAMP (Exton *et al* 1972, Rosberg *et al* 1974) Under basal *in vivo* conditions the release of cAMP gives a relatively constant concentration of cAMP in the blood although the turnover of cAMP in plasma is rapid (Chase and Aurbach 1967) Thus a continuous supply of cAMP

to the plasma is counterbalanced by an elimination of cAMP from the plasma Only a s ⌐l part of the eliminated cAMP is excreted via the kidneys (Broadus *et al* 1970) The low *in vitro* hydrolysis of cAMP by the blood also suggests that blood phosphodiesterases play only a minor role in the elimination of cAMP (Hemington *et al* 1973) It is however not known which tissues in the body that contribute to the major degradation of the extracellular cAMP It has been shown for some tissues that exogenously added cAMP can be degraded *in vitro* (MacManus *et al* 1971 Szabo and Burke 1972 Woo and Manery 1973) Of importance in this respect is that many enzymes participating in nucleotide metabolism are localized to the plasma membrane In fact it has been shown that one of the enzymes in nucleotide metabolism, 5 nucleotidase degrading 5 adenosine monophosphate (AMP) to adenosine is available only for extracellular substrate (De Pierre and Karnovsky 1974)

From studies with incubated frog muscles Woo and Manery (1973) have presented evidence that the degradation of exogenous cAMP takes place extracellularly and that this degradation is stimulated by insulin *in vitro* Since it is known that some endocrine diseases are associated with altered extracellular levels of cAMP the characterization of the extracellular cAMP degradation system(s) deserves special attention It is thus possible that hormones might directly regulate membrane localized enzymes in the nucleotide metabolism and thereby affect the levels of regulator substances like cyclic nucleotides with recognized importance for the cell metabolism In a forthcoming study evidence will be presented that FSH *in vitro* stimulates membrane bound phosphodiesterase activity in the prepubertal rat ovary (Selstam and Rosberg 1975)

The aim of the present study was to see whether exogenous cAMP is degraded and to characterize such a degradation by enzyme kinetics and by determining degradation products by paper chromatography in two isolated but intact organs the perfused rat heart and the incubated prepubertal rat ovary The release of cAMP during hormonal stimulation is much greater from the incubated prepubertal rat ovary (Selstam *et al* 1974) than from the perfused rat heart (Isaksson to be published) and it was considered of interest to compare these tissues We have in this study measured the degradation of cAMP at a concentration of 10^{-4} M or lower since estimated concentrations of cAMP in most extracellular fluids do not exceed these levels Preliminary reports on part of this study have been published elsewhere (Rosberg *et al* 1974 Selstam *et al* 1975)

Materials and Methods

Chem als

The (^{32}P)-cAMP (100–2 000 Ci mol at experime tal date 96–99 purity) and (^{3}H-8)-cAMP (7 5 Ci, mmol 98–99 purity) were purch sed from The Radiochemical Centre Amersham (^{14}C 8)-5 ad nosine monophosphate (C AMP 5 5 Ci mol 98–99 purity) was obtained from the New England Nuclear Co

Rad oact e substances except ^{32}P-cAMP were purif ed by paper chromatography when less than 99 pure All other chem cals including AMP adenosine, aden ne and theoph ll ne were of analytical grade and purchased from Merck or S gma Chemical Co's.

Exp n ntal proc dures

Perf on of hearts Hearts from fed female Sprague–Dawley rats, weighing 140–160 g were used The ra were anesthetized with ether and the hearts were rap dly exc sed and transferred to ice-ch lled

The aorta was cannulated and retrograde perfusion with Krebs bicarbonate buffer at 37 C from a reservoir 70 cm above the heart was started immediately After a 5 min washout period the hearts were perfused for 90 min as a modified Langendorff preparation with recirculating Krebs bicarbonate buffer with half the normal cal ium concentration (1 25 mM) and containing glucose (? 5 mg/ml) ^{32}P-cAMP (0 1 1 0 μM) and ^{3}H-cAMP (0 1–1 0 μM) In some experiments ^{14}C AMP (1 mM) was added The recirculating volume was 10 0 ml at the start of the perfusion period and it was continuously gassed with 95 O_2 and 5% CO_2 equilibrated with water The temperature of the perfusate was maintained at 37 C and the perfusion pressure was 60 mm Hg For further details concerning the heart perfusion technique see Isaksson (19) and Morgan *et al* (1961) At different time periods during the perfusion 0 1 ml aliquots of the perfusion medium were withdrawn in duplicates from the coronary effluent with a micropipette To one al quot $ZnSO_4$ and $Ba(OH)_2$ were added for Ba Zn precipitation (see below) and to the other water in equal amounts The present sampling technique made it possible to follow the changes in the metabolism of exogenously added labelled cAMP and its degradation products in the perfusion medium with time At the end of the perfusion period the hearts were frozen between blocks of aluminium cooled to the temperature of liquid N_2 The hearts were stored at −80 C until analyzed

Incubation of ovaries Sprague–Dawley rats 23–24 days old weighing 45–49 g were used They were starved for 18– 4 h before the experiments The rats were sacrificed by cervical fractures and the o aries rapidly removed and placed in ice-chilled buffer Each ovary was carefully trimmed free from extraneous tissues under a stereomicroscope rinsed blotted on filter paper weighed on a torsion balance and placed in a "preincubation" flask Only ovaries weighing between 6 and 9 mg were used The preincubation me ium consisted of 1 ml Krebs bicarbonate buffer with half the normal calcium concentrat on (1.25 mM) and containing glucose (1 mg ml) The ovaries were preincubated for 60 min at 37 C under continuous shaking and with 95 O_2–5 CO_2 as gas phase The ovaries were then transferred to 15 ml glass tubes and incubated under the same conditions in 0 5 ml medium with the addition of ^{3} P-cAMP (0 0 5 1 0 μM), ^{3}H-cAMP (0 0.5 1 0 μM) and in some experiments AMP adenosine adenine or theophylline at a con centration of 1 mM Incubation periods lasted for 60–1 0 min and at the end of the incubations the o aries were immediately frozen in Frigen 11® chilled with solid carbondioxide and stored at −80 C until analyzed.

T eatment of t ssues and media

A piece of the frozen left ventricle of the heart (15–40 mg) or one intact ovary was homogenized in 1.5 ml 5 trichloroacetic acid and centrifuged The supernatant was extracted three times with ether a dried overnight and redissol ed in 0 5 ml water To 0 1 ml aliquots of these tissue extracts" and also to media samples equal amounts of 0 15 M $ZnSO_4$ and 0 15 M $Ba(OH)_2$ were added according to the method descr bed by Krishna t al (1968) After centrifugation (0 000 g for 70 min) 10 ml of Insta Gel (Pa k ard) was added to 0 1 ml aliquots of the supernatant and the radioacti ity was measured in a Packard Tricarb Sc nt llat on Spectr meter (Model 33 0) To al quots of non precipitated tissue and media sam les water was dded n equal amounts and radioactivity was measured in the same way The quenching was checked w th ext rnal standards and was found to be equal for all samples within an experime t Cor rections wer made for th P-decay Krishna *et al* (1968) reported that $ZnSO_4$ and $Ba(OH)_2$ com letely precip tated n p e ept cAMP and adenosine In the present investigation less t an 0 1 of label ed cAMP w f u be precipitated under the experimental conditions used Adenos ne was fou d to be preci el 15 The following substances were found to be completely precipi tated by ZnS(t ' t enosine triphosphate (ATP) 5 adenosine diphosphate (ADP), AMP inorganic phos nosine hypoxanthine and cyclic 3 ,5 guanosine monophos hate (cGMP) The t carried out at 0–4 C

Paper chromatog

Al quots of 0 0 m d Ba Zn precipitated ti sue extracts and med a sam les were applied as narrow No 1 paper (4 cm 46 cm) Descend g chromat grams were developed for 1 h w M mmonia – M acetic acid (100 19 1) pH 7 15 a cord g to Osnes *et al* (19) In way To all chromatograms ap ro imately 5 moles of unlabelled cAMP AMP plied and of these purines the pos tions af er development were visualized by u tra para e experiment the posi ions of ATP ADP and aden ne were also determined. T were then cut into 2 cm segments and separately placed in counting ials in such a w red the walls of the vials and 12 ml of In ta-Gel was added. The sc lla on Pard was 10 t mes CPM values were corrected f r o erlapping and t e up

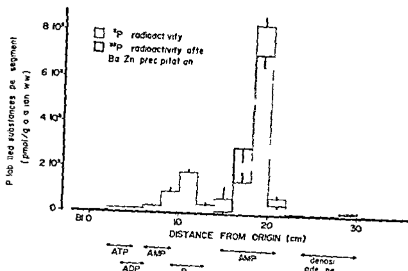


Fig 1 Distr bution of ³²P rad oact vity in ovarian incubat on medium determined by paper chromatography Aliquots of non treated and Ba–Zn precip tated m dia from ovaries incubated for 2 h in 0 25 μM ³ P-cAMP were chromatographed on Whatman paper No 1 Descending 36 cm chromatograms were run fo 1 h with 96 ethanol M ammon a 2 M acetic acid (100 19 21) pH 7 15 The chromatograms were cut in cm segments which were counted in a liqu d scintillation spect ometer The amounts of labelled substances per segment were calculated as described in Methods Each histogram represents mean alues of segments from 5 chromatograms. S E. is indicated by a vertical bar The pos tions of chromatographed unlabelled ATP ADP AMP cAMP adenosine and adenine as v sualized by ultra violet light are shown in the lowe part of the figure

radioactivity for decay The relati e yield in the paper chromatograms (total CPM for an 0 0 al quot applied to a segment CPM fo an equal al quot diluted directly in 12 ml of Insta-Gel) was for ³²P radioactivity 99 ⁴C radioactivity 73 % and for H radioacti ty 15 A typical chromatogram with the distribut on of ³²P labelled substances is shown n Fig. 1

Calculations and stat i al p oced e

D gradation of ³²P-cAMP and uptak of H and P ad oa ti i) The rate of P-cAMP degradation was determined by measuring the disappearance rate of rad oact vity f om Ba Zn-precipitated perfus on and incubation med a The uptake of H and P labelled substan es was measured as disappearance of radioacti ity from non precip tated media Degradat on and uptake were corrected for d lution of rad oactivity into the extra-cellular space olume as determined by sucrose (Hjalmarson and Isaksson 1972 Nilsson and Sebstam 1975) The degradation and uptake of labelled substances based on the d sappearance of rad oactivity from the med um was calculated using the following formula

$$[CPM_{t_0} - CPM_t ((V+ec)/V)] S \quad V (CPM_{t_0} \quad w)$$

where CPM = r d o ct ty n non precip tated or Ba Zn-precip tated media samples at the ndicated time t_i V = perfu ion or incubation olume ec = extr cellular olume S = concentration of cAMP at zero time, w tissue wet weight.

Distribut on of labelled sub tances in ch omatograms The amounts of labelled substances in segments from a chromatogram were calculated by mult plying the percentage of H and ³²P rad oact ty in the segments with the calculated amounts of labelled substances in the chromatographed med um or t ssue The distribution of labelled substances in the tissue was corrected for radioactiv ty in the extracell space by assuming an equal d stribution of labelled substances in the med m and in the extracell space

Stat stical pro dure Mean alues are gi en ± S E. Comp risons were mad by Student s a p valu of 0 05 or less was considered s gn ficant in th s study

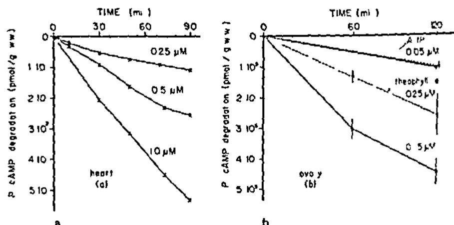


Fig 2. Degradation of extracellular ^{32}P-cAMP by rat heart (a) and prepubertal rat ovary (b) Rat hearts were perfused with 10 ml Krebs bicarbonate buffer containing glucose (5 mg ml) and ^{32}P-cAMP (0 5–1 0 µM) Aliquots of the perfusion medium were taken during the perfusion ^{32}P-cAMP contents in the media were determined by measuring the radioactivity after Ba–Zn precipitation The values for each concentration presented are from individual hearts Prepubertal rat ovaries were incubated in 0.5 ml Krebs bicarbonate buffer containing glucose (1 mg ml) 0 05 µM ^{32}P-cAMP with or without unlabelled AMP (1 mM) and 0 25 µM ^{32}P-cAMP with or without theophylline (1 mM) Ovaries were incubated for the times indicated and ^{32}P-cAMP degradation in the media was determined as described above Each point represents the mean value of 5 ovaries S E. is indicated by a vertical bar

Results

It will be seen from Fig 2 that both the heart and the ovary degraded ^{32}P-cAMP in the perfusion and incubation media respectively The rate of degradation of ^{32}P-cAMP was found to decrease somewhat at the end of the perfusion and incubation periods probably due to the decreasing concentrations of labelled cAMP in the media with time On increasing the initial concentration of cAMP in the medium the rate of degradation of cAMP was increased (Fig 2 a and b) The rate of degradation was decreased approximately 50 when theophylline at a concentration of 1 mM was added to the ovarian incubation media when the initial concentration was 0 25 µM (Fig 2 b)

In order to study enzyme kinetics the degradation rates for different concentrations of ^{32}P-cAMP were determined Rat hearts were perfused with ^{32}P-cAMP at different concentrations (0 1–1 0 µM) and samples from the perfusion medium were taken at 0 10 30 50 70 and 90 min after the start of perfusion Since fairly straight lines were obtained when the velocities for the degradation of ^{32}P-cAMP between the sampling times were plotted versus time initial velocities for the different concentrations were achieved by extrapolating to zero time In Fig. 3 inverted values of the calculated initial velocities are plotted versus inverted substrate concentrations (Lineweaver and Burk 1934) A straight line calculated with the method of least squares (regression coefficient r = 0 99) gives an apparent K_m of 1 95 µM and an apparent V_{max} of 315 pmol g w w min for this phosphodiesterase activity of the rat heart Fig 4 demonstrates a Lineweaver Burk plot for phosphodiesterase activity of the incubated prepubertal rat ovary Ovaries were incubated for 120 min with ^{32}P-cAMP at different concentrations (0 025 1 0 µM) and the velocity for each concentration was cal

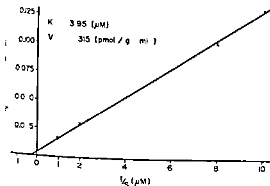
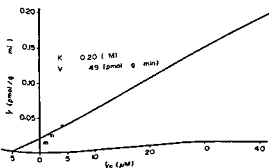


Fig 3 Lineweaver Burk plot of phosphodiesterase activity in perfused rat heart Rat hearts were perfused with different ^{32}P cAMP concentrations (0 1–1 0 μM) as described in Methods Aliquots were taken from the media at diff rent times (10–90 min) and ^{32}P-cAMP content was determined by Ba–Zn precipitation The calculated initial velocities pre ent d in the figure were estimated by extrapolatin, the velocities between consecuti e sampl ng times for each concentration to zero time The regression line was calculated by the method of least squares with a regression coefficient of 0 996 based on observations from 6 individual hearts

culated For the concentration range of 0 025–0 25 μM a straight line was calculated by the method of least squares giving an apparent K_m of 0 20 μM and a V_{max} of 49 pmol/g w w × min The two highest concentrations tested (0 5 and 1 0 μM) were not used in these calculations of kinetics since a deviation from linearity for these concentrations was a consistent finding in several experiments In preliminary experiments indication of a phosphodiesterase enzyme with a higher K_m value has been found which might explain the deviation from linearity when the concentration of cAMP was increased above 0 25 μM

Both the heart and the ovary accumulated ^{3}H and ^{32}P labelled substances In the heart the uptake was fairly equal for both labelled substances (Fig 5 a) while in the ovary the uptake of ^{3}H labelled substances was much more pronounced (Fig 5 b) The rate of uptake of ^{3}H labelled substances was for both tissues found to be fairly linear with time and more pronounced with increasing ^{3}H cAMP concentrations (Fig 5) In the above mentioned experiments the uptake was calculated on the disappearance of radioactivity from the medium When the uptake of labelled substances was calculated on the radioactivity in tissue extract of the heart with correction for the extracellular space the uptake after 90 min of perfusion with 0 5 μM labelled cAMP was 604 and 510 pmol labelled substances/g wet weight for H and ^{32}P radioactivity respectively (*cf* Fig 5 a) This difference in results between the two methods of determining uptake of labelled substances is probably due to



F g 4 Linewea er Burk plot of phosphod este ase act vity in incubated prepubertal rat o ary Prepubertal rat o ar es were incub ted with d fferent P-cAMP concentr t ons (0 0 5–1 0 μM) for 120 min as described in M thods At the end of th incubat on per od the P-cAMP content of the incubat on med a was determ ned by Ba Zn prec pitation The regression line was calcul t d by the method of least sq ares nd the r gres ion coeffic nt was found to be 0 984 when the v l s for the two h ghest o ce tratio (0 5 / M d 1 0 / M) w re e l ded Lach point represe ts th me n of 5 o ar es

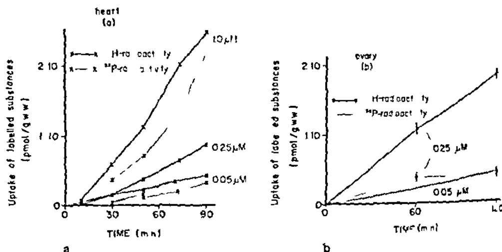
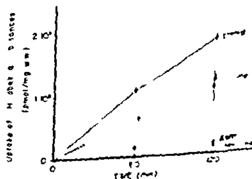


Fig 5 Uptake of ^{3}H and ^{32}P labelled substances by rat heart (a) and prepubertal rat ovary (b) Rat hearts were perfused with 10 ml Krebs bicarbonate buffer containing glucose (2 5 mg/ml) and ^{3}H and ^{32}P-cAMP (0 25–1 0 μM) Aliquots of the perfusion medium were taken during the perfusion Prepubertal rat ovaries were incubated in 0 5 ml Krebs bicarbonate buffer containing glucose (1 mg/ml) and ^{3}H and ^{32}P-cAMP (0 05 and 0 25 μM) Uptake of ^{3}H and ^{32}P radioactivity was determined by measuring the disappearance of ^{3}H and ^{32}P radioactivity from the medium as described in Methods For the ovary each point represents the mean value of 5 ovaries S E is indicated by a vertical bar

loss of radioactivity during weighing and freezing of the heart A similar difference although smaller was also noticed for the ovary

To determine whether or not the degradation of cAMP to AMP occurred extracellularly unlabelled AMP was added to the ovarian incubation media at a concentration of 1 mM As may be seen from Fig 6 AMP effectively inhibited the uptake of ^{3}H labelled substances, suggesting that cAMP was degraded extracellularly (Fig 6) The degradation of ^{32}P-cAMP was unchanged in the presence of AMP (Fig 2 b) AMP seemed also to be degraded extracellularly since unlabelled adenosine (1 mM) effectively inhibited the uptake of ^{3}H labelled substances In contrast to AMP and adenosine unlabelled adenine (1 mM) inhibited the uptake only moderately In order to see whether the phosphodiesterase activity was bound to the tissue or could be found in the medium ovaries were first incubated for 4 hours

Fig. 6. Uptake of ^{3}H labelled substances by prepubertal rat ovary with and without added AMP adenosine adenine or theophylline Ovaries were incubated in 0 5 ml Krebs bicarbonate buffer containing glucose (1 mg/ml) and ^{3}H-cAMP (0 5 μM) and incubated for the periods indicated The test substances were added at a concentration of 1 mM Uptake of ^{3}H radioactivity was determined by measuring the disappearance of ^{3}H radioactivity from the medium as described in Methods. Each value represents the mean of 5 ovaries. S E is indicated by a vertical bar



without labelled cAMP Ovaries were then removed from the media and ^{32}P-cAMP was added to the medium (final concentration 1 μM) and the media were incubated for an additional 2 h The ^{32}P-cAMP degradation in these media was less than 5% compared to media containing ovaries It was not possible with the present procedures to find out whether the degradation of labelled cAMP in the medium by the heart occurred extracellularly since it was found with chromatographic analysis that 1 mM ^{14}C AMP was completely degraded after only 10 min of perfusion

Distribution of radioactivity as determined by paper chromatography of perfusate and tissue extracts from rat hearts perfused with 0 5 μM ^{3}H and ^{32}P-cAMP are shown in Table I The amounts of ^{3}H and ^{32}P-cAMP found in the tissue after 90 min of perfusion corresponded very closely to the expected amounts in the extracellular space demonstrating that the concentration of intracellularly located labelled cAMP must have been close to zero The main part of ^{3}H labelled substances in the tissue extract was found in the adenosine adenine region of the chromatograms (Table I) Small amounts of ^{3}H labelled substances were found in the ATP ADP region and in the AMP region ^{32}P labelled substances were found in the ATP ADP AMP and P_i regions

Aliquots of the perfusion medium were withdrawn at different periods of time and chromatographed to study the change in the amounts of labelled cAMP and its degradation products (Table I) Only small amounts of ^{3}H labelled substances were found apart from ^{3}H cAMP In the adenosine adenine region a fairly constant amount was seen throughout the perfusion period and seemed largely to consist of adenosine since approximately 60% remained after Ba–Zn precipitation ^{32}P labelled substances were found in all regions indicated in Table I In the AMP region a fairly constant amount was found during the perfusion period while in the ATP ADP region and to an even greater extent in the P_i region an accumulation in the perfusion medium was seen with time An impurity of the ^{32}P cAMP preparation (1–2%) found in the adenosine adenine region of non perfused medium (0 min Table I) remained in the medium during perfusion The pattern of distribution of ^{3}H and ^{32}P labelled products with time was the same for all concentrations of cAMP (0 1–1 0 μM)

At all times studied a difference between the amounts of ^{3}H and ^{32}P-cAMP was seen in the rat heart perfusion medium (Table I) The observed degradation rate of ^{32}P-cAMP was approximately twice as high as that of ^{3}H-cAMP suggesting recirculation of tritiated compounds to ^{3}H-cAMP during the perfusion This difference between ^{3}H and ^{32}P-cAMP degradation was seen for all concentrations of cAMP (0 1–1 0 μM) tested

Table II demonstrates the pattern of degradation products in ovarian tissue extracts and incubation media after incubation periods of 60 and 120 min in the presence of 0 25 μM ^{3}H and ^{32}P-cAMP In contrast to the heart the rate of disappearance of ^{3}H and ^{32}P-cAMP was similar in the ovarian incubation media. ^{3}H and ^{32}P labelled substances in the tissue were found in the ATP ADP AMP and adenosine adenine regions Small amounts of ^{32}P labelled substances were found in the ATP ADP AMP and P_i regions The degradation products in the medium from ^{3}H-cAMP were found in the AMP region and to a greater extent in the adenosine-adenine region of the chromatograms In the latter region approximately 2/3 was precipitated by Ba–Zn precipitation suggesting that aden

TABLE II Degradation of labelled cAMP in the medium and distribution of radioactivity in incubation medium and ovarian tissue determined by paper chromatography Prepubertal rat ovaries were incubated for 60 and 120 min in 0.5 ml Krebs bicarbonate buffer containing glucose (1 mg ml) and ^{3}H and ^{32}P-cAMP (0.25 μM) After the incubation periods samples of the incubation media and tissue extracts were chromatographed before and after Ba–Zn precipitation The table shows the degradation of cAMP in the medium and the distribution of radioactivity in medium and tissue The amount of radioactivity in the tissue was calculated from the disappearance of radioactivity in the medium at the end of the incubation period The distribution of radioactivity in the tissue has been corrected for amounts of radioactivity in the extra cellular space of the ovary (30 of the wet weight) assuming the concentration of labelled products in the extra-cellular space to be the same as in the medium. Values presented represent means ± S E of 5 ovaries.

	cAMP-degradation in the medium[a] (pmol labelled cAMP/g ovarian wet weight) × 10^{-3}		Amounts of labelled substances in media and tissues (pmol labelled substances g ovarian wet weight) × 10^{-3}					
			Unprecipitated					Ba Zn precipitated
	Unprecipitated	Ba–Zn precipitated	0–6[b] ATP ADP	6–10 AMP	10–14 P_i	16–.. cAMP	.4–30 Adenosine adenine	.4–30 Adenosine
^{3}H-radioactivity								
Medium, incubation time (min)								
60	4.71 ± 0.70	3.65 ± 0.51	N D	0.6. ± 0.13	N D	—[d]	2.74 ± 0.46	0.94 ± 0.15
120	5.48 ± 0.46	4.89 ± 0.43	N D	0.75 ± 0.25	N D	—[d]	2.71 ± 0.23	0.99 ± 0.15
Tissue, incubation time (min)								
60	—	—	0.47 ± 0.05	0.14 ± 0.0	N D	N D	0.33 ± 0.04	N D
120	—	—	.03 ± 0.18	0.58 ± 0.07	N D	N D	0.80 ± 0.09	N D
^{32}P-radioactivity								
Medium, incubation time (min)								
60	3.86 ± 0.73	3..1 ± 0.47	0.33 ± 0.05	0.68 ± 0.13	.34 ± 0.38	—[d]	0.35 ± 0.03	0.29 ± 0.0
120	5.48 ± 0.70	4.78 ± 0.46	0.39 ± 0.03	0.96 ± 0.15	3.43 ± 0.50	—[d]	0.33 ± 0.0.	0..8 ± 0.0.
Tissue, incubation time (min)								
60	—	—	0.1. ± 0.0.	0.04 ± 0.01	0.06 ± 0.01	N D	N D	N D
120	—	—	0.1. ± 0.04	0.03 ± 0.01	0.07 ± 0.03	N D	N D	N D

[a] cAMP degradation was measured as initial radioactivity minus 16–.. cm parts of chromatograms.
[b] Regions of chromatograms (cm from origin) with the positions of chromatographed unlabelled purines and the position of phosphate.
[c] Not Detectable. These values were not significantly different from zero
[d] Not shown due to differences in ovarian weight.

and Burke 1972) and rat thymic lymphocytes (MacManus *et al* 1971) also degrade extra cellular cAMP These results thus imply that several tissues participate in the metabolism of extracellular cAMP and probably can account for the fact that the main part o cAMP in the plasma is eliminated by factors other than renal clearance. In the present study the elimination of cAMP by both the heart and the ovary obeyed enzyme kinetics. The apparent K_m value for the heart was found to be 3.95 μM K_m values of 0 8 μM and 3 85 μM have been reported for phosphodiesterase activities in homogenates and purified enzyme preparations respectively from the heart (Beavo *et al* 1970 Thompson and App

In these as well as in other studies (Pöch 1971 Goren and Rosen 1971 Hrapchak and Rasmussen 1972) phosphodiesterase activities with K_m values of 25–500 μM were reported A high K_m value (55 μM) was also found for extracellular cAMP degradation in the frog muscle (Woo and Manery 1973) It is of interest in this connection that phosphodiesterase activity with high K_m values can also have an affinity for another cyclic nucleotide cGMP (Beavo *et al* 1970 Russel *et al* 1973)

For the prepubertal rat ovary the degradation of exogenously added cAMP to the incubation medium gave an apparent K_m value of 0 2 μM Phosphodiesterase activity with approximately the same K_m value as well as K_m values of 50 250 μM have been found in homogenates of corpora lutea and prepubertal ovaries from the rat in studies from this laboratory (to be published) Stansfield *et al* (1971) reported a high K_m value (250 μM) for homogenized bovine corpora lutea The K_m values for the heart and the ovary demonstrated by the present investigation thus agree fairly well with those found in homogenates of these tissues From the kinetic experiments in the present study it is obvious that the ovary degrades cAMP more rapidly than the heart at low extracellular concentrations of cAMP This observation implies that the more pronounced accumulation of cAMP in the incubation media from the ovary after stimulation with LH (Selstam *et al* 1974) than in the perfusate of the heart after stimulation with adrenaline (Isaksson to be published) when the level of intracellular cAMP in the two different tissues is increased to approximately the same level cannot be explained by differences in degradation of exogenous cAMP between these tissues

The degradation of cAMP from the medium might either be due to an uptake and subsequent degradation of cAMP intracellularly or to a degradation of cAMP by extracellularly active enzymes For the ovary the degradation of cAMP was not inhibited when AMP or adenosine at a high concentration was added to the incubation medium although these substances almost completely inhibited the uptake of degradation products of cAMP strongly suggesting that cAMP was degraded extracellularly The possibility that the degradation of cAMP in the medium was caused by enzyme which had leaked out into the medium was ruled out in control expriments (see Results) The elimination of cAMP from the ovarian incubation medium thus seems to consist of a degradation of cAMP by phosphodiesterase which is attached to the cell surface Similar observations have previously been reported by Woo and Manery (1973) using incubated frog skeletal muscle For the heart it was not possible with the technique used to dilute effectively degradation products of cAMP by addition of AMP since AMP was rapidly cleared from the perfusate The question thus still remains whether the disappearance of cAMP from the heart perfusate is due to extracellular degradation of cAMP or to an uptake of cAMP with a subsequent intracellular degradation

The degradation products of cAMP found in the ovarian incubation medium and heart perfusate included phosphorylated and dephosphorylated compounds In the heart perfusate both P_i and adenosine were found indicating that AMP was further degraded to adenosine The pattern of degradation seems to be the same in the ovary although the rate of AMP degradation proceeded more slowly than in the heart In contrast to the pattern of degradation of AMP in the heart and the ovary found in the present study AMP was

degraded to 5 inosine monophosphate (IMP) and inosine in frog skeletal muscle demon strating that the pattern of AMP degradation differs between tissues and/or species (Dunk ley *et al* 1966 Manery *et al* 1968) It cannot however be excluded from the present ex periments that IMP and inosine might be intermediate metabolites in the degradation of AMP

The rate of disappearance of ³H-cAMP from the heart perfusate was approximately half the rate of ³ P-cAMP disappearance The difference can probably be explained by .. re-cycling" of cAMP *i e* tritiated degradation products of ³H-cAMP were taken up by the myocardial cells and incorporated into ATP Tritium labelled cAMP is subsequently formed and released to the perfusate Since no detectable amounts of ³H-cAMP could be identified intracellularly the proposed release of ³H-cAMP must have been very rapid or cAMP must have been produced in close association to the cell membrane and then released The recycling hypothesis is supported by other observations demonstrating that adenosine is very rapidly taken up by the heart and incorporated into adenine nucleotides (Jacob and Berne 1960) In the ovary direct proof was obtained that adenosine was a degradation prod uct of cAMP taken up by the cells since added adenosine but not adenine completely inhibited the uptake of ³H labelled substances

It is at present premature to more extensively speculate about the physiological impor tance of the degradation of extracellular cAMP Changes in the plasma levels of cAMP however accompany some endocrine diseases (for ref see Broadus *et al* 1971 Hardman *et al* 1971 Karlberg *et al* 1974) which might be due to changes in the activity of extra cellularly active phosphodiesterase enzymes The question whether different hormones di rectly influence the activity of these enzyme(s) will therefore be of importance in forthcom ing studies For one hormone insulin, evidence has been elicited that this hormone directly stimulates membrane bound phosphodiesterase enzymes (Woo and Manery 1973 Thomp-son *et al* 1973) It is however possible that membrane bound phosphodiesterase enzymes are most important for membrane and intracellular processes by having their substrate sites mainly towards the cell interior and that the degradation of extracellular substrates only reflects part of the characteristics of the enzyme A hypothetical function of extra cellular cAMP might be that this nucleotide acts as an intercellular messenger within organs composed of many cell types such as the ovary The activity of membrane bound phos phodiesterase enzymes would consequently be of significance for this hypothetical function

We are indebted to Professor Kurt Ahrén for valuable d scuss ons Excell nt technical as istance was given by Miss Britt Marie Johansson and M ss Stina Öberg.
This invest gat on was supported by grants from the Swedish Medical Research Cou c l (B74 14X 50-0.. B75 14X 27 11) the Medical Faculty Göteborg, and Magnus Bergvalls Stiftelse

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man and dog is indeed SP To our knowledge this is the first demonstration that SP like material is present in blood

The present study was supported by grants from the Swedish Medical Research Council (project no 04X 3571 and 04X-4495)

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